HUMAN GROWTH HORMONE

A Methodological
and
Clinical Study

by Otto Westphal

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by

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PART I

Radioimmunoassay of Human Growth Hormone

CHAPTER I

Introduction

Until recently the assay of hormones in blood was achieved in most instances by bio-assay techniques. A number of bio-assays have been suggested for human growth hormone (HGH) (Evans and Simpson 1931, Marx et al. 1942 and Evans et al. 1943). However, the specificity and precision of all the biological methods were insufficient for them to be of clinical use. Read and Bryan (1960) adapted the haemagglutination-inhibition test for HGH, which proved to be satisfactory for the quantitative determination of HGH in highly purified anterior pituitary extracts but was unreliable when applied to human serum because of nonspecific factors in the serum. The radioimmunological method for insulin was introduced in 1959 by Berson and Yalow and was applied to HGH independently by Hunter and Greenwood (1962 a), Utiger et al. (1962) and Glick et al. (1963). The principle of the method depends on the competition between radioactively labeled and unlabeled hormone for the specific binding sites of the antibody (Fig. 1). The higher the concentration of the unlabeled hormone the less radioactively labeled hormone will be bound to the antibody. After separating the bound from the free hormone, the fractions can be measured. Using suitably small, known quantities of unlabeled hormone a standard curve is prepared. The concentration of hormone in unknown samples can be read off the curve directly.

In the radioimmunoassay of hormones in blood it is customarily assumed that labeled and unlabeled hormones behave identically, that the circulating hormone is chemically and immunologically identical with the standard hormone preparation, that the amount of hormone bound by the antibody is independent of the concentration of the hormone and that different antisera made against a particular antigen will give a similar quantitative response in the immunoassay system.

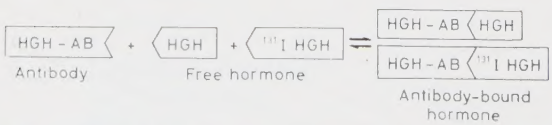

Fig. 1. Principle of radioimmunoassay.

The HGH used for immunoassay has been prepared by several different methods (Li and Papkoff 1956, Raben 1959, Roos et al. 1963) and there seems to be some differences in homogeneity between the preparations. It would be more precise to use the term immunoreactive growth hormone for HGH amounts estimated with radioimmunoassays. This term however is inconvenient to use in practice and HGH is substituted in the following for immunoreactive growth hormone.

The various methods available for radioimmunoassay of HGH differ mainly in the technique used to separate free from antibody-bound hormone. A perfect system would show no labeled antigen in the "bound" fraction in the absence of antibody and 100 per cent of the labeled antigen in the "bound" fraction after incubation with excess antibody. A number of methods for separation have been published, among them, by electrophoresis on cellulose acetate membranes, (Hunter and Greenwood 1964), polyacrylamide gel electrophoresis (Fitschen 1964), chromatoelectrophoresis (Glick et al. 1963), ion-exchange-resin (Lazarus and Young 1966), adsorption on charcoal (Lau et al. 1966) and by using a second antibody to precipitate the antibody-bound hormone (Utiger et al. 1962, Hartog et al. 1964 a, Ceraci et al. 1966).

In the present study special interest has been directed to the chromatoelectrophoretic method described by Glick et al. (1963). This method was originally published for insulin by Berson et al. (1956). Small amounts of HGH are strongly adsorbed on a special paper whilst that bound to antibody moves towards the anode with the gamma-globulins. The separation must be carried out at 4°C since the heat generated by the current may cause dissociation of the labeled HGH at higher temperatures. Yalow and Berson (1964) however showed that the flow of buffer along a strip may be effected very simply by evaporation and that method has been used in this study. The method is described in detail and the influence of damage to the labeled HGH has been studied. Special attention is given to the application of the method on neonatal plasma.

CHAPTER II

Analytical procedure for a radioimmunoassay of human growth hormone

In collaboration with Paul Roos* and
Jan I. Thorell**.

Source of human growth hormone

HGH prepared by the method of Roos et al. (1963) was used throughout the study without further purification for immunisation, for labeling and as standard HGH. HGH-I.C.R.F. batch 6 peak II contributed by Dr. F. C. Greenwood, London and NIH-GH-HS 612 A obtained from Dr. A. Wilhelmi, Atlanta, Georgia have been used for methodological studies.

During the study six different Roos preparations have been used for immunoassay purposes. For the preparation of the guinea pig antiserum to HGH GF:19 was used. As standard solutions 1963 — 4/1965 GF 25:26:3 were used, later GF 29:2, and for methodological studies, GF 126. For labeling purposes no preparation older than 8 months was used, as it was noted that prolonged storage resulted in a decreased yield in the labeling procedure. The following preparations were labeled: GF 25:26:3, 29:2, 39:2, 48, 53 and for methodological studies, GF 57 and GF 60. The preparations have been lyophylised and stored at +4°C. Labeled growth hormone was prepared every 3—4 weeks. Before a new batch of growth hormone was used for labeling, its immunological activity was compared with the current laboratory standard. Furthermore the old and the new batches were labeled simultaneously to compare the immunological activity of the labeled preparations. No difference could be detected between the preparations except for preparations 57 and 60.

Preparation of ^{131}I-labeled growth hormone

The modification of Benerjee and Gibson (1962) of the original method by Hunter and Greenwood (1962 b) was used for labeling. The carrier free sodium (^{131}I) iodide was used within 36 hours of the commercial preparation. A 5μg HGH sample was shaken 60 sec with 2 mC sodium iodide and 30 μg chloramine-T as oxidizing agent. The reaction was stopped by adding 200 μg sodium meta bisulfite. All reagents except ^{131}I were dissolved in buffer 1. For buffer solutions see below.

The separation of ^{131}I-labeled HGH from the reaction mixture has been modified from the Banerjee-Gibson (1962) method and from that used by Hunter and Greenwood (1962 b). The mixture was passed through a column of Sephadex G-50 (Pharmacia, Uppsala, Sweden), the column measuring 1.8 cm in diameter and containing 10 g of dry gel. The gel was allowed to swell in barbital buffer 2 in a beaker for 24 hours before packing of the column. To minimize adsorption onto the gel, 100 mg crystalline serum albumin was passed through the column followed by a minimum of two column volumes of buffer 2 prior to

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the application of the reaction mixture. The columns were run gravimetrically at a flow rate of approximately 3 ml/5 minutes. Fractions of 1½ ml were collected manually. To the fractions 0.05 ml of 7 % bovine serum albumin in buffer 3 was immediately added in order to prevent radiation damage. They were then counted in a well-type scintillation counter (Philips PW 4119) with suitable shielding.

The radioactivity was obtained in 3 peaks (Fig. 2). The approximate yield of labeling was calculated by the relation between the salt peak and the protein containing peaks.

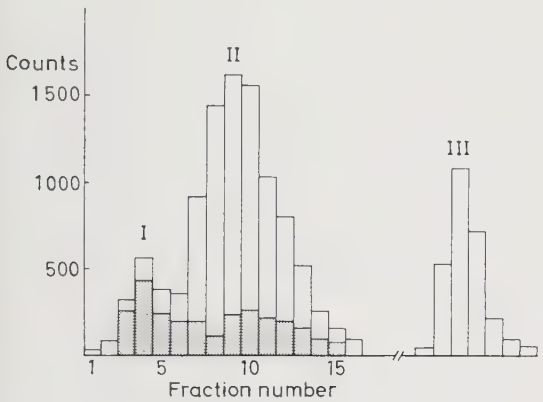

Fig. 2. Gel filtration of reaction mixture of ^{131}I-labeled growth hormone. Counts are arbitrary radioactive units. ⁙ indicates amount of damaged hormone. The best fractions are in the front part of the second peak. In this experiment fraction 8 was used for immunoassay. Peak III is the radioactive I-131 salt peak.

All fractions were tested for degradation products (damaged HGH) in the following way: 10 µl of gel-filtration fractions were mixed with 100 µl of a 7 % bovine serum albumin solution in buffer 2 and 500 µl of buffer 3. Fifty µl of this mixture was applied on a moistened 2×25 cm paper strip (Whatman 3 MC). A minimal amount of Brom Cresol green was dropped at the application line for flow identification. The paper strips were suspended horizontally with one end immersed in buffer 2. Intact HGH is adsorbed at the application line while the degradation products migrate with the carrier proteins in the buffer flow to the center of the paper strip. This modification of the originally chromato-electrophoretic technique of Berson et al. (1956) was chosen since it was shown by Yalow and Berson (1964) that the flow of buffer along the strip may be effected very simple by evaporation. If antibody is added the antibody-bound growth hormone and the damaged labeled hormone move together in the buffer-flow chromatography as well as in the chromato-electrophoretic system. Flow chromatography was performed at room temperature not exceeding 20°C using cooled buffer 2. The procedure was stopped after about 30 minutes using the front of the Brom Cresol green as indicator for adequate separation. After drying the strips at 120°C they were scanned with a Geiger-Müller tube using a slit of 2 mm. The separation is shown in Fig. 3.

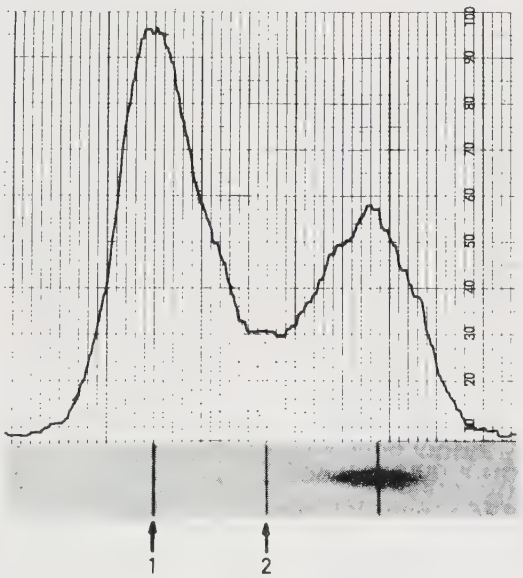

Fig. 3. Buffer-flow chromatograph on paper strip and the monitored activity.
↑ 1 indicates application site. ↑ 2 indicates line where cutting of the strip was performed. The dark zone is the Brom Cresol dye indicating the fraction that moves with the proteins.

The fractions of damaged HGH in different tubes was calculated. The total fraction of damage (D) during the labeling was cal-

culated in the following way: d = sum of counts of damaged HGH in all tubes with more than 100 counts in peaks I and II. (Shadowed area in Fig. 2) T = sum of counts in all tubes with more than 100 counts in peak I and II. $D = \frac{d}{T} \times 100$. The total damaged fraction D was usually about 25 %. The tube with the least fraction of damage was used for the assay. It was diluted in buffer 3 to a concentration of 10 ng/ml and kept at —20°C.

Human growth hormone standard

During the period 1963 to April 1965 a standard solution was prepared from Roos GF 25:26:3. Stock solutions in concentrations 1, 10 and 50 ng/ml in buffer 3 were prepared and kept at —70°C. The amount sufficient for one month's use was kept at —20°C. An accident with the main part of the standard solutions made it necessary to prepare a new standard. A freshly prepared Roos HGH, GF 29:2 was diluted and stored as above. Very careful tests with repeated standard curves could not reveal any differences in the immunological activity of the two preparations.

Guinea pig antiserum to HGH

Adult guinea pigs were injected subcutaneously three times with 1 mg of Roos GF:19 in Freunds complete adjuvant. The intervals between the injections were three weeks, the guinea pigs were bled by cardiac puncture one week after the last injection. The serum and stock dilutions were kept at —70°C, amounts for one month's use, at — 20°C. Antiserum Ma 1 was used throughout the study. This antiserum, in antibody excess proportions completely bound ^{131}I HGH. The binding was fully prevented if the antibodies were saturated with unlabeled HGH.

Buffer solutions

1. For preparation of ^{131}I HGH: 0.16 M borate buffer pH 8.0. 2. For gel filtration and buffer-flow chromatography: 0.1 M Barbital buffer (LKB 3276) pH 8.6. 3. For dilution of antibody and ^{131}I HGH, for HGH-standard solutions and as incubation diluent: 0.1 M Barbital buffer (LKB 3276) with 0.25 % crystalline bovine serum albumin.

Reagents and equipment

Indicator for buffer-flow indentification: 2 ml 0.1 M Barbital buffer (LKB 3276) pH 8.6 with 7 % crystalline bovine serum albumin and 2—3 grains of Brom Cresol green (Tetra brom-m-cresolsulfonephthalein).

Albumin: Crystalline bovine serum albumin (BSA) was a gift from AB Kabi, Stockholm, Sweden. It was prepared by Armour and Co. Eastbourne, Sussex, England.

^{131}Iodide: Sodium ^{131}I, carrier free (IBS3). The Radiochemical Centre, Amersham, England.

Pipettes: For pipetting labeled HGH, HGH-antibody and plasma into incubation tubes: Hamilton microliter syringes with 100 µl capacity and 5 cm cemented needle and Chaney-Adaptation (710—NCH) from Hamilton Company, Whitteer, Calif. USA. For HGH-standards: AGLA brand Micrometer Syringe Outfit from Burroughs Wellcome & Co London, England.

Chromatography paper: Whatman 3 MC. Two different batches without significant differences were used throughout the study. (Four of six tested batches were not suitable).

Counting equipment: Philips PW 4119 well-type scintillation counter and Baird Atomic automatic sample changer.

Incubation mixture

In a 5.5×1.0 glass tube a mixture of:

1. 0.2 ng ^{131}I HGH.

2. 50 µl plasma or various amounts of HGH-standard giving final concentrations of 0.1, 2, 10, 20, 50 and 100 ng HGH/ml.
3. Guinea pig antiserum to HGH, final dilution 1:500.000.
4. Buffer 3 used as diluent to a final incubation volume of 500 µl.

For the standard curve duplicate tubes were prepared for every concentration of standard HGH. For plasma samples triplicate tubes were prepared.

In order to enable calculation of the incubation damage an incubation mixture identical with the standard or unknown plasma sample, but without antibody, was prepared in duplicate for the standard curve and in triplicate for plasma from each patient. If there were more than 3 samples from the same patient the first and last samples were tested. The incubation mixture was mixed carefully after preparation and 24 hours later. It was kept at +4°C for 4 days.

Separation of free and antibody-bound HGH

To every tube for the standard curve 50 µl of adult heparin plasma (from blood donors) was added as carrier protein. To the plasma samples 50 µl buffer 3 was added to keep identical volumes. After moderate shaking 2×125 µl was put on a 4×25 cm paper strip of Whatman 3 MC paper previously moistened with buffer 2. On the application site a minimal amount of Brom Cresol green was added as indicator. Flow chromatography was then performed at room temperature not exceeding 20°C, using buffer 2 that was kept at +4°C by adding pieces of the frozen buffer. The procedure was identical with that described for testing the labeled HGH fractions. The chromatography was stopped when the front had moved exactly 6 cm (after about 30 min.). This gave an adequate separation between the free HGH that was adsorbed at the application site and the antibody-bound HGH that moved with the protein. The strips were dried at 120°C and cut in two parts between application site and the moving proteins and the radioactivity measured. The control tubes (incubation mixture without antibody) were handled identically.

Calculation of standard curve

By this method it is possible to make an approximate correction for damaged HGH which in the buffer-flow system moves with the protein and the antibody-bound HGH but has no antibody binding capacity. The calculation is made on the control samples (incubation mixture without antibody). If I is intact HGH measured at the application line and M is damaged HGH moving with the protein and the per cent damaged is K than $K = \frac{M}{M+I} \times 100$.

The true $\frac{B}{B+F}$ ratio of a sample is, if F is the free HGH measured at the application point and B_1 is antibody-bound and damaged HGH moving with the proteins:

$$\frac{B_1 - K\ (B_1+F)}{F+B_1-K\ (B_1+F)}\ .$$

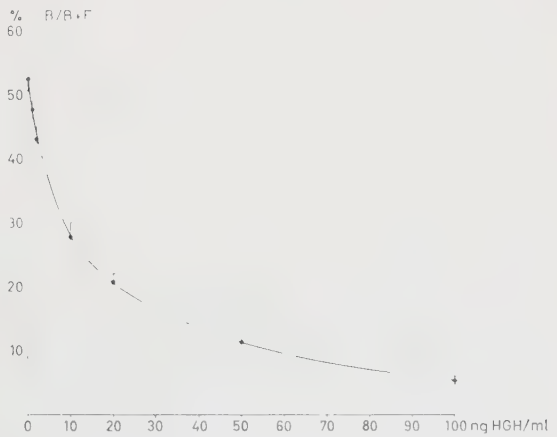

Fig. 4. Mean and S.D. of 10 consecutive standard curves plotted in a linear diagram. Bars indicate S.D.

K varies between 10—30 % and if 35 % or more the precision of the method is markedly diminshed. In the standard curve the true $\frac{B}{B+F}$ ratio is expressed in per cent. The standard curve can be plotted in a linear system with per cent $\frac{B}{B+F}$ on the ordinate and the HGH concentrations on the abscissa, (Fig. 4). It is, however, more convenient to plot the curve in a semilogarithmic diagram, (Fig. 5), since the curve is nearly linear between 2 and 100 ng HGH/ml.

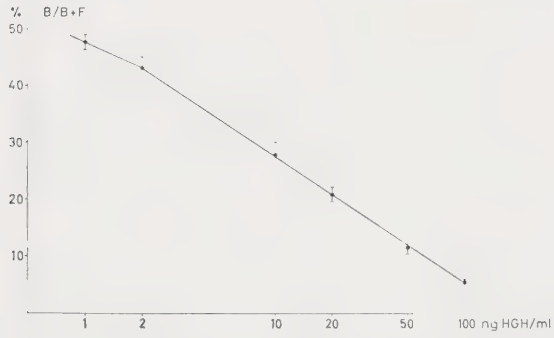

Fig. 5. Mean and S.D. of 10 consecutive standard curves plotted in a semilogarithmic diagram. Bars indicate S.D.

Calculation of test sample

This was performed in the same way as the standards and the $\frac{B}{B+F}$ ratio was compared with the standard curve, plotted in the semilogarithmic system.

A standard curve and 25—35 test samples in triplicate and with controls as above can be analysed in one series. The laboratory routine was to prepare samples and run buffer-flow chromatography twice a week.

CHAPTER III

Methodological studies of the immunoassay for HGH

Effect of different HGH preparations as standard HGH

All HGH preparations used for assay routine are prepared by the method described by Roos et al. (1963). The preparations used are stated in chapter II. So far however no international standard preparation of HGH for immunoassay purpose is available. To compare the laboratory standard with other preparations the following experiments were performed. (November 1966).

HGH preparations studied were:

1. Roos GF:6 prepared 1962 and kept lyophylised at +4°C.
2. Roos GF 29:2 our laboratory standard for immunoassay. Stock solutions in buffer 3 and in concentration 1, 10 and 50 ng/ml were prepared within a month after the preparation of the hormone and kept at —70°C. No previously thawed samples were used as standards in these experiments.
3. A British HGH preparation (Imperial Cancer Research Fund batch 6, peak II) contributed by Dr. F. C. Greenwood. This sample was prepared 1964 and, since December 1964, was kept dissolved in buffer 3 and stored as Roos GF 29:2.
4. A lyophylised Wilhelmi preparation NIH —GH—HS 612 A.

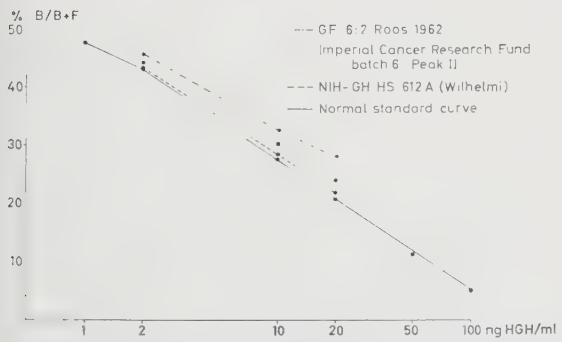

Fig. 6. Standard curves with different HGH preparations.

The lyophylised preparations were dissolved in barbital buffer 3 one week before the experiment. The results are shown in Fig. 6.

The Roos HGH consists of at least four different components when separated on column electrophoresis as shown in Fig. 7.

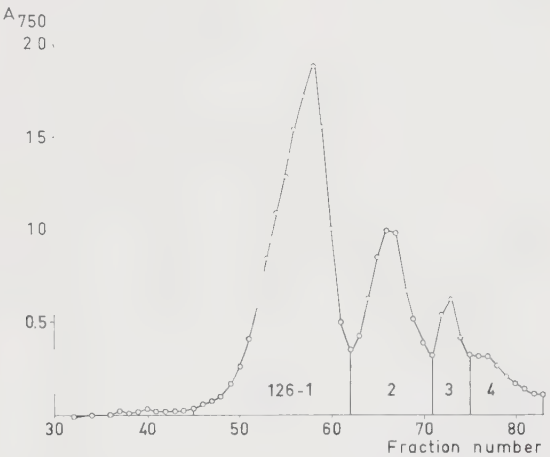

Fig. 7. Protein distribution from column electrophoresis at pH 8.6 of HGH obtained through gel filtration on Sephadex G-100.

These components have identical biological activity (Roos et al. 1963) and similar lactotrophic activity (Lions et al. 1966). The immunological activity of the four components has been studied in a second experiment. The fractions 126:1—4 were components of GF 29:2. The results in Fig. 8 indicate that fraction 126—4, which constitutes a minor

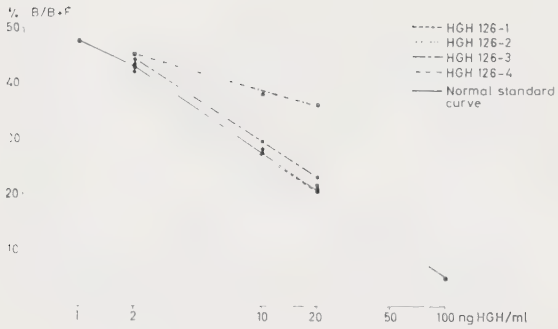

Fig. 8. Immunological activity of four different electrophoretic components of GF:29 compared with normal standard curve of GF:29.

portion has an immunological activity markedly lower than the other components.

From the first experiment it can be concluded that the HGH assay of a given sample can differ if compared with different standard preparations. The immunological activity of our laboratory standard is higher than the preparation used by Greenwood and slightly higher than the Wilhelmi preparation. From this it follows that our HGH values will be lower than those published by authors using one of the above mentioned preparations as standards. This indicates the necessity of an international standard for HGH used for immunoassay. The markedly low immunological activity of GF:6 (Fig. 6) might be the result of the storage of the preparation but might even indicate that earlier Roos preparations were less active.

The second experiment shows that available "clean" growth hormone easily can be fractionated into different components and despite the fact that these components have identical biological activity the immunological activity varies. From this it can be concluded that the immunological and biological sites of the HGH molecule are not completely identical. Thus HGH preparations intended for clinical purposes must be tested not only for their immunological activity but for their biological activity as well.

Separation of ^{131}I labeled HGH from reaction mixture

Initially the method of Hunter and Greenwood (1962 b) was tested. The radioactivity was monitored in two peaks, the protein peak and the salt peak. There was no apparent difference in the amount of degradation damage in the different tubes of the protein peak and since the degradation damage often exceeded 30 % a more proper separation was necessary. Gelfiltration was chosen as a very mild method. Different amounts (2.1—17.5 g) of Sephadex G-50 and G-75 were tried in 1.8 cm diameter columns. The reaction mixture was diluted to a total volume of 2 ml and 0.5 ml was used for every column. The Sephadex used was allowed to swell in buffer 2 in a beaker for 24 hours, and following packing of the column 10 mg crystalline serum albumin per gram Sephadex in buffer 2 was run through the column to prevent adsorption of labeled protein. A minimum of two column volumes of buffer 2 was passed through the columns prior to the application of the reaction mixture. All columns were run gravimetrically, the fractions were collected manually in volumes of 1.5 ml. The results obtained in 2 different labeling procedures are shown in Table I.

As seen in Fig. 9 a double protein peak is seen when 10 g or more of Sephadex G-50

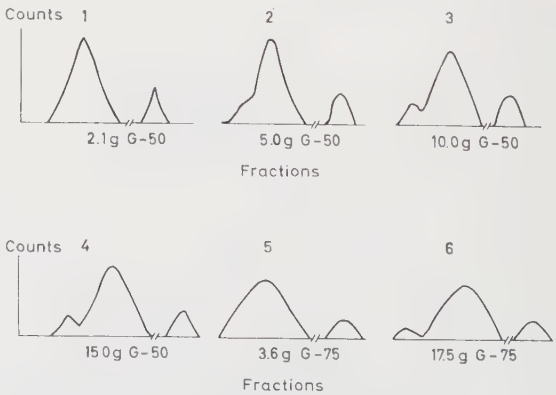

Fig. 9. Approximate separation of labeling mixture by gel filtration on varying amounts of G-50 and G-75.

TABLE I. *Effect of variations in Sephadex separation of reaction mixture*

Mode of separation	Lengt of column in cm	Per cent damaged in best tube	Recovery of reaction mixture	Labeling
Sephadex G-50 2.1 g	10	27	81 %	
Sephadex G-50 5 g	23	22	80 %	1
Sephadex G-50 10 g	46	12	78 %	
Sephadex G-50 15 g	70	11	78 %	
Sephadex G-50 2.1 g	10	29	79 %	
Sephadex G-50 10 g	46	12	77 %	2
Sephadex G-75 3.6 g	7	50	83 %	
Sephadex G-75 17.5 g	35	19	79 %	

is used. There was no advantage in using more than 10 g G-50 or using G-75. The separation with 10 g G-50 was chosen, usually giving a labeled product with less than 15 % degradation damage in the best tube.

Some factors influencing the degradation damage

The problem of degradation products has been dealt with by several authors (Yalow and Berson, 1964, Midgley, 1966 and Glover et al. 1967) and is one of the main difficulties in the labeling procedure.

In our laboratory as in others the labeling procedure has failed in periods, the only variable being a new preparation of ^{131}I. Generally a bad labeling was characterized by a poor yield (less than 40 %), and a large amount of degradation products. Such preparations could not be used for assay purposes. It was early experienced that the ^{131}I had to be freshly prepared, thus it was normally used within 24 hours after the commercial preparation, and never later than 36 hours after the preparation.

For routine labeling lyophylised Roos GF 25:26:3, 29:2, 39:2 48 and 53 were used. No significant differences could be shown between the preparations used for the assay. The yield of the labeling procedure was usually between 60—90 % giving a specific activity of 240—330 μc/μg, the preparation containing less than 1 atom of iodide per molecule HGH.

Since the problem of degradation damage is more pronounced for HGH than for other polypeptide hormones it is conceivable that the HGH molecule is more susceptible than other polypeptides. However, HGH is more heterogeneous than other hormones (Hunter 1965, Berson and Yalow 1966, Trygstad 1967). The heterogeneity of the Roos preparation has already been demonstrated (Fig 7). Furthermore it has been shown that lyophylising Roos' preparations gives rise to further heterogeneity which may be due to polymerization (Hansson et al. 1966).

To determine whether differences in the storage conditions of Roos HGH alters the labeling product two different batches of HGH prepared by the same procedure were labeled. In one experiment a lyophylised and a frozen (in buffer 1) preparation of GF 57 were labeled simultaneously. In a second experiment GF 57 lyophylised and GF 60 never lyophylised nor frozen were labeled. The results are listed in Table II. The

TABLE II.
Effect of storage of HGH on labeling

Preparation	Per cent yield of labeling	Sum of damaged in % of total protein (D)
GF 57 lyophylised	68	21.8
GF 57 frozen	60	18.1
GF 57 lyophylised	70	20.3
GF 60	65	12.3

yield of the labeling procedure seems to increase on lyophylising the preparations. On the other hand, the less manipulation with the hormone the fewer degradation products. This could indicate that the degradation damage in part is present already prior to the labeling and that this damaged fraction is more easily labeled by ^{131}I than is intact HGH. Further evidence for this will be published elsewhere (Westphal et al. 1968).

Testing of antisera

Four different antisera were prepared simultaneously by the procedure described earlier. They were tested in the following way. Fifty µl of labeled HGH with a calculated concentration of 10 ng/ml was incubated with the antisera in final dilution of 1:10,000, 1:100,000, 1:250,000, 1:500,000, 1:1,000,000 and 1:1,500,000 the total volume being 500 µl. The dilution giving a $\frac{B}{B+F}$ ratio of 50 % was chosen for mixing a standard curve with standard HGH amounts corresponding to 2, 10, 50 and 100 ng/ml. The standard curves showed almost identical slopes except for Ma 3, Fig. 10. For immunoassay purposes and throughout this study antiserum Ma 1 in final dilution 1:500,000 was used.

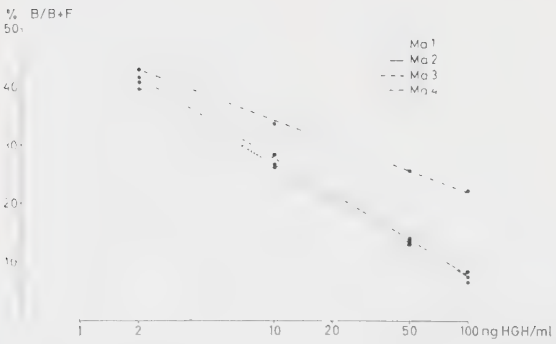

Fig. 10. Standard curves determined for 4 different guinea pig antisera.

Effect of different concentrations of unlabeled HGH on the binding of labeled HGH (standard curve)

Fig. 11 shows the mean and S.D. of ten consecutive standard curves plotted in a semilogarithmic diagram. Since the standard curve is very nearly linear between 2—100 ng/ml HGH, it is very convenient to use this system.

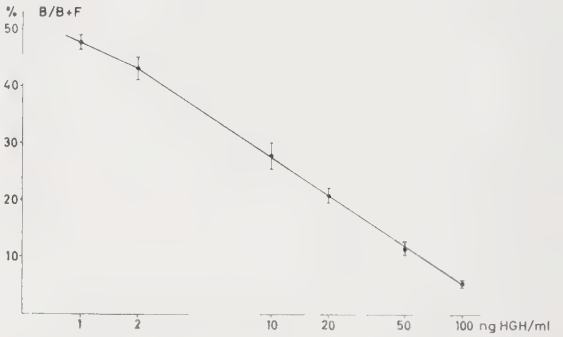

Fig. 11. Mean and S.D. of 10 consecutive standard curves plotted in a semilogarithmic system. The HGH standard was GF 29:2. Labeled hormone was GF 29:2 labeled on two different occasions and GF 39:2 labeled once. Bars indicate S.D.

Effect of different amounts of labeled HGH

The $\frac{B}{B+F}$ ratio will increase if the amount of labeled HGH is decreased. (Fig. 12). However, since very small amounts of labeled hormone make the counting procedure inconvenient that amount of labeled HGH giving a B/B+F ratio of 50—55 % with the antiserum in final dilution 1:500,000 has been used for routine assays. This has been checked on every freshly labeled hormone by incubating a standard curve with 4 different amounts of ^{131}I HGH as shown in Fig. 13. For routine assays it gives a sufficient sensivity and a convenient number of counts.

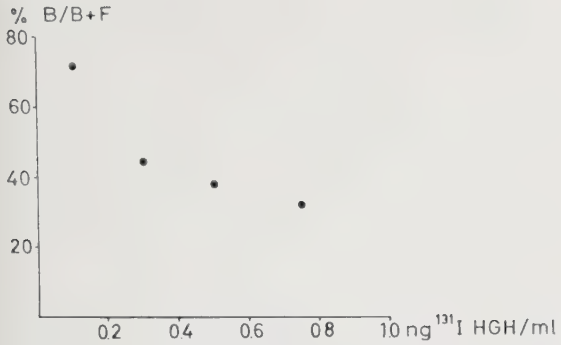

Fig. 12. B/B+F ratio without added standard HGH but various amounts of labeled hormone. Incubation time four days.

It is possible to increase the sensitivity of the assay by reducing the amount of labeled hormone, antiserum and consequently of standards. In a more diluted system however the equilibration time for the antigen-antibody reaction increases. If incubation time exceeds 7 days it is inconvenient. For special purposes however a more sensitive as well as a less sensitive system was prepared, Fig. 14.

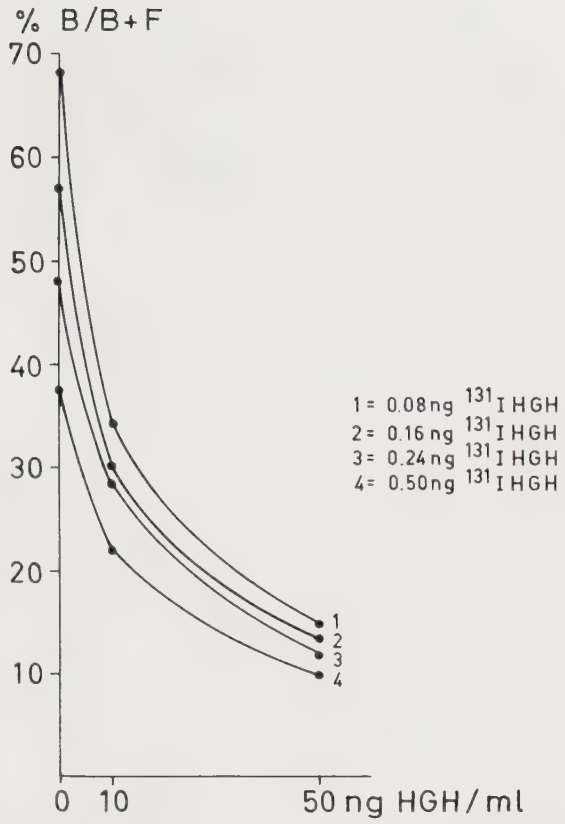

Fig. 13. Standard curves with various amounts of ^{131}I HGH. Dilutions as shown in the figure were made on every freshly labeled ^{131}I HGH preparation. The amounts giving a B/B+F ratio of 50—55 % was used for the assay.

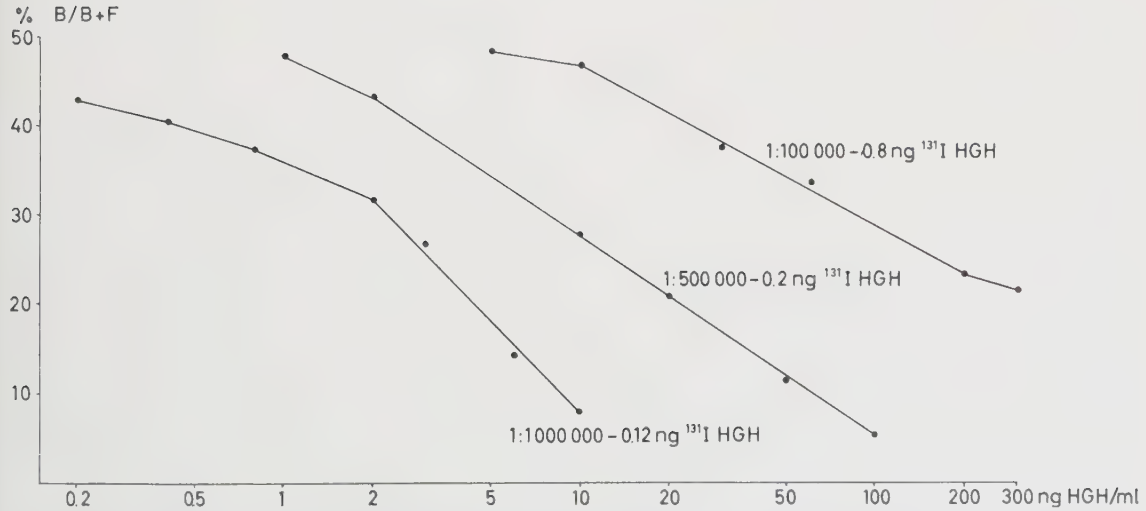

Fig. 14. Standard curves with various sensitivities of the assay. The concentrations of antisera were final dilution 1:100 000, 1:500 000 and 1:1 000 000. The corresponding amounts of ^{131}I HGH were 0.8, 0.2 and 0.12 ng respectively. Incubation time was 3, 4 and 7 days respectively.

Comparison between standard HGH and labeled HGH

On several occasions during the study the standard curve was compared with another obtained when labeled HGH was used as a standard. Fig. 15 shows no significant differences in the immunological behaviour of the two standards.

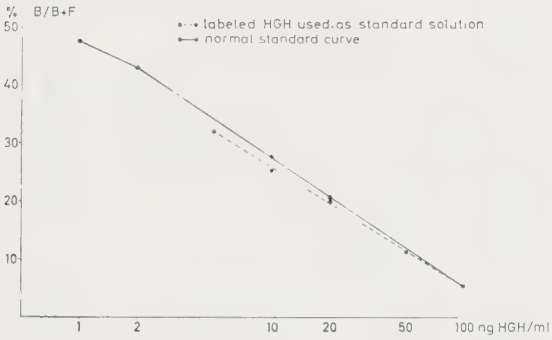

Fig. 15. Standard curves with laboratory standard GF 29:2 and with labeled GF 39:2.

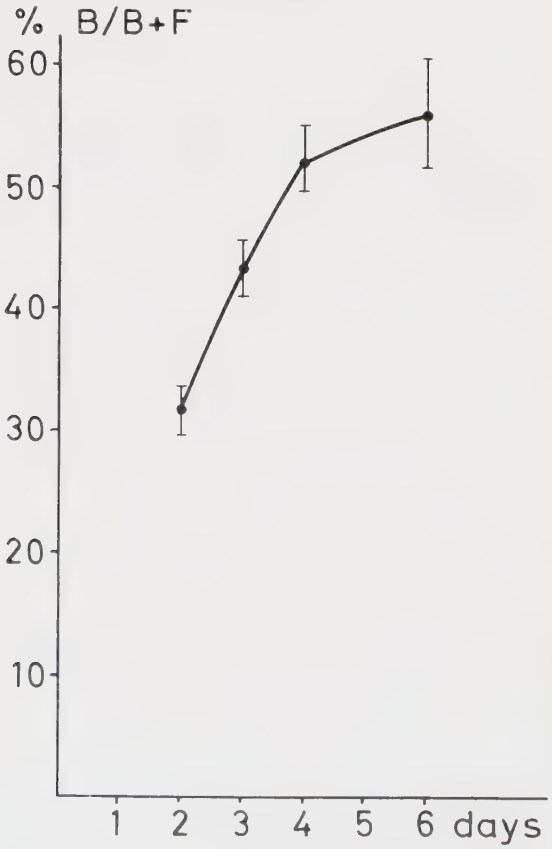

Fig. 16. Effect of incubation time. Bars indicate S.D.

Effect of incubation time on binding of labeled HGH

For this experiment GF 29:2 was used for labeling. The amount of ^{131}I HGH was 0.15 ng/500 µl incubation mixture. Incubation periods of 2, 3, 4 and 6 days were studied and the results are given in Fig. 16. No equilibrium in binding occurred during this period but the difference between four and six days was almost negligible. The S.D. was greater in the six day incubated sample. The reason for this was an increase in the incubation damage. For the assay an incubation time of 4 days was routine.

Effect of protein on standard curve

Differences in the effect of plasma on the assay have been published. Hunter and Greenwood (1964) have stated that if plasma is diluted 1:5 or more no unspecific binding will occur while Cerasi et al. (1966) show an unspecific binding in their double antibody assay. For this purpose three different standard curves were mixed.

1. Ordinary standard curve without protein during incubation.
2. Standard curve incubated with 50 µl of 7 % crystalline bovine serum albumin in barbital buffer 2.
3. Standard curve with 50 µl of heparinized bovine plasma.

As shown in Fig. 17 no significant differences are noticable. Thus it is not necessary to incubate the standard curve with protein.

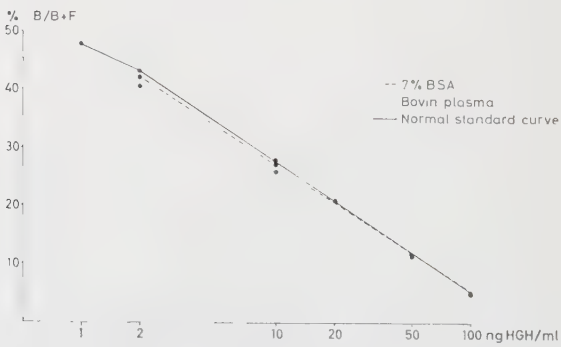

Fig. 17. Effect of protein in incubation mixture on standard curves. Incubation time four days.

Effect of degradation damage on standard curve

The precision of the radio-immunoassay decreases as the amount of damaged hormone increases (Berson et al. 1964). Since the amount of damage increases with prolonged storage or by storage of the labeled hormone at room temperature (Berson and Yalow 1966), four standard curves were run simultaneously with the same labeled hormone, the hormone being stored in different ways. The results are shown in Table III.

Furthermore, three samples of the same HGH preparation (GF 39:2) were labeled on separate occasions and measured when one was one, another four, and the third six weeks old. The preparations were kept at —20°C and had not been thawed prior to the experiment. The results are shown in Fig. 18.

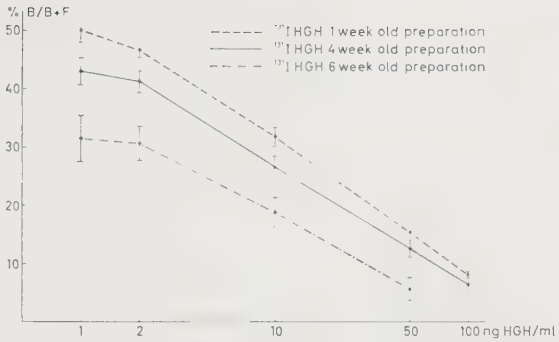

Fig. 18. Labeled HGH GF 39:2 from different labelings and with approximately the same amount of damage at time of preparation. The preparations contained degration damage of 17 %, 36,5 % and 49 % respectively at the time of the experiment. Bars indicate S.D.

TABLE III. *Effect of storage of labeled HGH*

Storage of preparation GF:39	Incubation damage	Per cent B/B+F ±S.D. for various amounts of standard HGH 1 ng/ml	2 ng/ml	10 ng/ml	50 ng/ml
Frozen	19 %	49±2.2	44±2.1	28±1.8	12 ±0.8
+4°C 24 hours	22 %	48±2.0	43±2.7	27±1.5	11.9±0.9
20°C 6 hours	30 %	46±2.6	41±2.7	26±1.9	10.8±1.2
20°C 24 hours	46 %	38±3.9	36±3.7	23±2.7	8.7±2.1

Both experiments show that the slope of the standard curve is diminished when the damaged fraction increases and that will decrease the precision and consequently increase the S.D. For assay purpose labeled hormone with a degradation fraction greater than 35 % has not been used. The labeling procedure was repeated every third or fourth week. The labeled hormone was kept at —20°C, it was thawed in a refrigerator at +4°C and was kept in an ice water bath during the mixing procedure. The incubation mixture was kept at +4°C until it was put on the paper strips for chromatography.

Further effects of temperature on the assay

During early experiments with the assay duplicates of the standard curve and triplicates of plasma samples showed a significant difference between comparable samples. It was also noted that on those occasions the time of the buffer-flow chromatography was prolonged and the variation between comparable strips pronounced. This effect increased during the day when the buffer used for the flow chromatography assumed room temperature. Since there was no sign of increase in degradation damage it was presumed that

temperature had a direct influence on the flow chromatography. An inadequate difference in temperature between buffer and surroundings presumably gave rise to an irregular rate of evaporation and buffer-flow. The temperature of the buffer was kept at +4°C with pieces of ice and the room temperature was not allowed to exceed 20°C. With these precautions the flow rate was rather constant and, the S.D. was diminished.

Cutting the paper strips

Initially the paper strips were monitored with a Geiger-Müller tube with a small slit, the surface of the two peaks was calculated by planimetry. This very time-consuming procedure was substituted by simply cutting the strips. They were then put in plastic tubes, placed in an automatic sample changer and counted. The cutting was performed exactly halfway between the application line and the buffer-flow indicator as seen in Fig. 3. To test the accuracy of this cutting the two methods were compared by running 100 strips by both methods. The difference never exceeded ±2 % of the B/(B+F)-ratio.

Effects of other hormones on the assay

To check whether other hormones may influence the assay 50 µl of two concentrations of ACTH, pituitary LH, pituitary FSH, chorionic gonadotrophin and insulin respectively were incubated 4 days with 0.2 ng ^{131}I HGH and HGH antiserum in final dilution 1:500,000. The results are shown in Table IV. Only LH of pituitary origin and in high doses has any influence on the assay. Probably the available LH preparation contained a small amount of HGH. The B/(B+F) ratio in samples with the other hormones tested did not exceed the B/(B+F) ratio of the standard tubes without any added standard HGH. This would have been the case if the available antiserum contained antibodies against the actual hormones.

TABLE IV. *Effect of some other hormones on the immunoassay of HGH*

Preparation	Amount in ng	Apparent amount of HGH ng/ml
ACTH*	50	—
	1000	—
LH**	50	—
	1000	18.3 ± 2.1
FSH**	50	—
	1000	—
Chorionicgonadotrophins***	50	—
	1000	—
Insulin****	50	—
	1000	—

*Cortrophin, AB Pharmacia, Uppsala, Sweden (Source: Porcine pituitaries).
**Kindly prepared by Paul Roos, Ph.D., Inst. of Biochemistry, Uppsala, Sweden.
***Gonadex Leo, Hälsingborg, Sweden. (Source: Urine of pregnant women).
****Novo AS Copenhagen, Denmark. (Source: porcine panchreas).

CHAPTER IV

Application of the method to human plasma with special reference to neonatal plasma

All blood samples were collected in heparinized glass tubes (Vitrum, Stockholm, Sweden), volume 10 ml. If not otherwise stated the assay was performed as follows. A mixture of 0.2 ng ^{131}I HGH, guinea pig antisera to HGH, final dilution 1:500,000, standard HGH in various amounts for incubation of standard curve as described earlier or 50 µl plasma and buffer 3 to a final incubation volume of 500 µl was incubated for 4 days. The samples within one experiment are all run in the same series if not otherwise stated.

During the whole investigation two pooled samples were run in every series. One pooled sample was obtained prior to exchange transfusion from 3 children 4 days of age. The other pool was fasting blood from 3 healthy children 11 years of age. The estimated values were 33 ±1.8 and 4.2 ±0.8 ng/ml (50 determinations).

Effect of incubation time

The pooled samples were incubated 3, 4 and 6 days, respectively. The results are shown in Table V. Prolonged incubation seems to result in lower measurable concentration of HGH, and greater S.D., the latter due to increased degradation damage.

Effect of amounts of labeled HGH and antisera to HGH

The pooled samples were incubated 4 days with amounts of labeled HGH and antisera as shown in Fig. 14 and compared with corresponding standard curves. The results are shown in Table VI. Differences in the obtained results are not noticeable.

TABLE V. *Effect of incubation-time*

	HGH levels in ng/ml ± S.D. (5 estimations)	
Incubation-time	Neonatal plasma	Adolescent plasma
3 days	35 ± 4.2	3.8 ± 1.0
4 days	33 ± 4.2	3.0 ± 0.9
6 days	30 ± 5.9	2.5 ± 1.7

TABLE VI. *Effect of amounts of labeled HGH and antisera to HGH*

^{131}I-HGH		HGH levels in ng/ml ± S.D. (5 estimations)	
Antisera	Incubationstime	Neonatal plasma	Adolescent plasma
0.8 ng / 1:100.000	3 days	34 ± 4.1	<5 but >0
0.2 ng / 1:500.000	4 days	33 ± 3.8	3.1 ± 0.9
0.12 ng / 1:1.000.000	6 days	>10	3.1 ± 0.8

Effect of storage on frozen plasma

The pooled plasma samples have been stored for a period of more than 2 years at —20°C. The values obtained after 5 days, 1 month, 1 year and 2 years are shown in Table VII. When the 5 days and 1 month samples were run, the laboratory standard was GF 25:26:3. For the remaining samples the laboratory standard was GF 29:2. No difference in obtained HGH values was noted after 2 years' storage at —20°.

TABLE VII. *Effect of storage of frozen plasma (—20°C)*

Time	HGH levels in ng/ml ± S.D. (5 estimations)	
	Neonatal plasma	Adolescent plasma
5 days	34 ± 4.2	2.7 ± 0.9
1 month	31 ± 4.0	2.9 ± 1.0
1 year	32 ± 3.8	2.4 ± 0.8
2 years	31 ± 4.1	3.2 ± 0.9

Effect of room temperature on whole blood and plasma

Freshly drawn blood samples from a 4 day old infant and from a healthy 11 year old girl were divided into two equal parts. One part was allowed to stand for various lengths of time at room temperature before it was spun down and the plasma separated and frozen. The other part was immediately spun and the separated plasma allowed to stand for various lengths of time at room temperature before being frozen. The results are shown in Table VIII. No noticeable changes are seen in plasma samples if stored 6 hours at room temperature. In whole blood, however, a decrease in immunoreactive HGH is detectable within one hour in neonatal blood and within 2 hours in adolescent blood. So the blood samples are immediately put in a refrigerator or in a beaker with ice and spun down and separated within one hour.

TABLE VIII. *Effect of room temperature on whole blood and plasma*

Time	HGH in ng/ml plasma. Mean ± S.D. of 5 estimations			
	Neonatal		Adolescent	
	blood	plasma	blood	plasma
10 min	20.2 ± 0.7	19.2 ± 1.2	3.6 ± 0.3	4.0 ± 0.5
20 min	19.5 ± 0.4	19.4 ± 0.9	3.2 ± 0.9	3.6 ± 0.3
60 min	18.3 ± 0.7	20.1 ± 1.3	4.0 ± 0.7	4.3 ± 0.5
2 hrs	17.2 ± 0.4	20.3 ± 1.2	3.2 ± 0.5	4.2 ± 0.4
4 hrs	16.9 ± 0.7	19.6 ± 1.0	2.7 ± 0.9	4.1 ± 0.6
6 hrs	16.1 ± 0.8	18.8 ± 0.8	2.0 ± 0.8	3.9 ± 0.5
14 hrs	13.2 ± 0.9	17.1 ± 0.9	1.6 ± 0.9	3.7 ± 0.4
24 hrs	8.5 ± 1.1	15.5 ± 1.0	1.0 ± 0.7	3.2 ± 0.5
48 hrs	5.2 ± 1.0	13.5 ± 1.1	<0.5	2.5 ± 0.6

Effect of freezing and thawing on plasma samples

Tubes containing 0.8 ml of pooled plasma were thawed and frozen various times. All samples were estimated in the same series. The results are shown in Table IX and show no effect of repeated thawing.

TABLE IX. *Effect of thawing and freezing on plasma levels of growth hormone*

Number of freezings	HGH in ng/ml plasma. Mean ±S.D. of 5 estimations	
	Neonatal plasma	Adolescent plasma
1	19.5±2.1	3.2±0.9
2	20.5±1.1	3.2±0.8
5	21.8±1.6	3.7±0.5
10	21.7±1.9	3.4±0.7

Effect of hemolysis on plasma levels of immunoreactive growth hormone

Freshly drawn blood samples, one cord blood, one from a healthy 11 year old child were divided into 2 parts, the major portion was spun, the plasma separated and frozen. The minor part was immediately frozen to receive 100 % hemolysis without adding any substances. Twentyfour hours later the samples were thawed and different amounts of the hemolyzed blood were added to the plasma samples. The degree of hemolysis was measured by the method described by Martinek (1966). Results in Table X show that a fairly high amount of hemolysis is tolerated without interfering with the analysis. Very large amounts however will result in decreasing levels of HGH as well as an increase in S.D. Several explanations for the decrease of estimated immunoreactive HGH values are possible. One possibility is dilution of plasma with the intracellular fluid since the amount of HGH in the cells certainly is lower than in plasma. Furthermore the hemoglobin might act as a blocking agent on labeled HGH, which would simulate a decrease of available ^{131}I HGH, and with constant amount of antiserum the B/(B+F) ratio would increase, giving a lower HGH value. More probably, however, the hemoglobin interferes with the buffer-flow chromatography. Hemoglobin moves away from the application line as do other proteins. Probably it is able to carry away some of the antibody-free intact ^{131}I HGH. This explanation also accounts for the fact that incubation damage as measured in control samples without antibody increases with increasing amount of hemolysis, giving an increase in S.D.

TABLE X. *Effect of hemolysis on HGH levels*

Per cent hemolysis	HGH in ng/ml plasma. Mean ±S.D. of 5 estimations		Per cent hemolysis
	Neonatal plasma	Adolescent plasma	
0.005	75± 5.2	4.5±1.2	0.004
0.3	72± 4.0	3.7±0.9	0.4
0.8	78± 4.8	4.8±0.8	0.8
2.4	72± 5.2	4.0±1.3	2.3
10	68± 6.8	2.7±1.9	9.8
65	56±10.2	1.5±2.0	60
100	32±15.1	<1.0±2.0	100

CHAPTER V

Evaluation of the method

Recovery of exogenous HGH

The recovery of added HGH to neonatal and adult blood was studied. The neonatal blood was drawn from a five day old infant of a diabetic mother, the adult blood from a 25 year old student. The blood samples were divided in two equal parts, one for recovery in blood, one for recovery in plasma. As for recovery in blood, the samples were rocked in room temperature 15 minutes after the addition of exogenous growth hormone and prior to the separation of the plasma. Plasma and blood samples were allowed to stand at room temperature for about 25 minutes including centrifugation time. The results are shown in Fig. 19 and are summarized in Table XI.

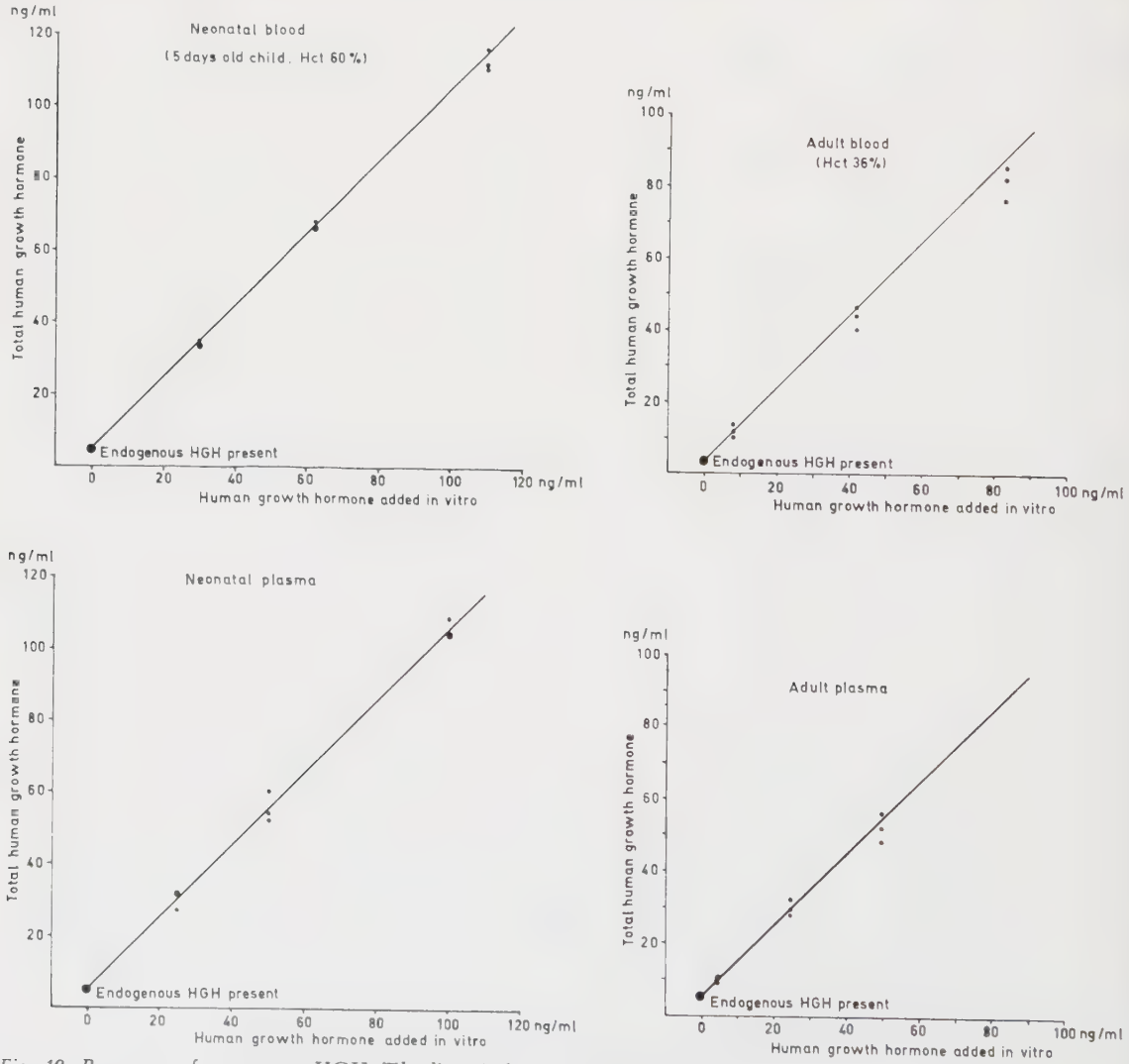

Fig. 19. Recovery of exogenous HGH. The lines indicate 100 % recovery.

TABLE XI. *Recovery of exogenous HGH*

Source	Per cent recovery (range)
Neonatal blood	97—107
Neonatal plasma	87—109
Adult blood	84—116
Adult plasma	92—110

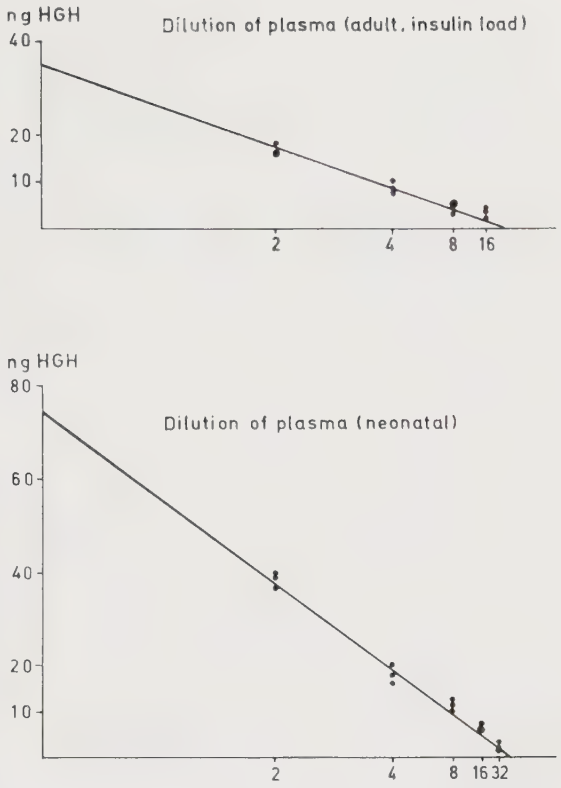

Fig. 20. Dilution of plasma. Lines indicate teoretical dilution if estimate of undiluted plasma is "true".

Reproducibility

The results of the estimation of the two pooled samples (page 23) run on 20 different occasions over a period of 9 months were compared. The mean and S.D. of the "between assay" estimations were 33.38 ±1.7 ng/ml for the pooled neonatal plasma and 4.26 ±0.76 ng/ml for the adolescent plasma. This study was followed by a "within" comparison where 20 samples of both pooled plasmas were run in the same series. The "within" mean and S.D. were 33.3 ±1.0 ng/ml and 4.2 ±0.58 ng/ml.

Accuracy of the assay

This has been studied in two ways.

1. Dilution of plasma. Plasma samples from an infant aged 6 hours and from a 25 year old student during insulin load were diluted serially. No attempt was made to correct for various amounts of protein during the incubation of the samples. However, for the buffer-flow chromatography the total amount of protein was kept constant by addition of various amounts of 7 % bovine serum albumin in 0.9 % saline. The results are shown in Fig. 20. If accuracy is defined as the per cent of "true" level, the accuracy range is 94—120 % for the adolescent plasma and 96—121 % for neonatal plasma. If the results are subjected to a student's t-test the variations do not significantly deviate from 100.

2. The 95 % confidence limits with determinations in triplicates were studied for plasma samples with respect to the variations in the standard curve. Such variations are not taken into account if the λ-value (S.D. of points around the regression line divided by the slope of the regression line) is estimated which has been used by some authors (Hales and Randle 1963, Cerasi et al. 1966). The 95 % confidence limits were about 20 % of the estimate which is in the same range as for other methods for immunoassay.

Sensitivity

The sensitivity of the assay is expressed as the lowest HGH concentration giving a significant decrease in B/(B+F) ratio. The comparison is made between the B/(B+F) ratio in tubes where no standard HGH was added and the B/(B+F) ratio which corresponds to 0.5, 1 and 2 ng/ml. In 20 consecutive standard curves studied, 14 showed differences between 0 and 1 ng/ml. All showed differences between 0 and 2 ng/ml ($p < 0.01$). So the sensitivity of the assay was on the level of 1 ng/ml. If the sensitivity was studied in the more sensitive system (Fig. 14) it was found to be on the level of 0.5 ng in all 10 standard curves studied.

CHAPTER VI

Clinical test of the method

The method has been tested on different groups of patients in order to compare the results obtained with those published by others.

Hypophysectomized patients

Two patients with certain total hypophysectomy for craniopharyngioma have been studied on several occasions after the operation. In all of totally 8 different plasma samples the obtained HGH level was <0.2 ng/ml when studied in the most sensitive assay used (page 19).

Acromegalic patients

The results are summarized in Table XII. The results agree with those published by Glick et al. (1963) and Hartog et al. 1964 a). Three acromegalic patients were studied during surgical hypophysectomy and the survival of endogenous HGH was observed. The results for two of the patients are shown in Fig. 21 and 22. The half-time for HGH for two of the patients was 24 and 28 minutes respectively. For the third patient surgical complications made the hypophysectomy very doubtful and that is proved by the half-time of 150 minutes. All patients showed some persistent HGH secretion following hypophysectomy. This is consistent with previous studies (Glick et al. 1964). The adherence of the capsule of the eosinophilic adenoma to surrounding blood vessels seems to make a total surgical extirpation impossible.

Fig. 21. Disappearance of growth hormone after hypophysectomy in a acromegalic patient. •—• indicates actual levels measured, o—o plasma levels after subtraction of residual constant concentration of 3 ng/ml. $T_{1/2}$ was calculated to be 24 minutes.

TABLE XII. *Fasting levels of HGH in acromegalic patients*

Source	No. of patients	Mean ± S.D. HGH ng/ml	Range HGH ng/ml
Acromegalic low activity	3	23 ± 12.1	12— 36
Acromegalic high activity	5	105 ± 26.5	71—138

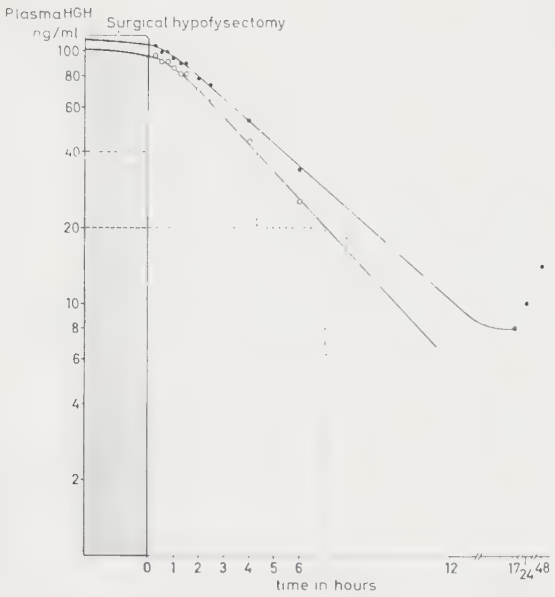

Fig. 22. Disappearance of growth hormone after hypophysectomy in a acromegalic patient. •—• indicates actual measurements, o—o plasma levels after subtraction of residual concentration 9 ng/ml. Calculated $T_{1/2}$ is 150 minutes. The hypophysectomy was complicated, the patient showed clinical signs of acromegaly one month after operation. HGH levels at that time, 76 ng/ml.

Cord blood and newborn infants

Samples of cord blood from 10 infants were studied. The mean level was 40.2 ±10.6 (S.D.). This is in agreement with the results published by Cornblath et al. (1965) and Laron et al. (1967). A special study of HGH levels in newborn infants is published in Part II.

Children and adults

Fasting levels of normal children and adults are very low and not uncommon below 1 ng/ml. Only a small series has been studied and the results are summarized in Table XIII. They are in agreement with the results of others (Greenwood et al. 1964).

It is of much greater interest to study the increase in HGH levels induced by hypoglycaemia, first shown by Roth et al. (1963 a). This has been studied in a series of 16 normal children. The results are discussed in detail in Part II. The HGH response to insulin-induced hypoglycaemia has been studied in 10 adults aged 22 to 47 years. The method was identical with that applied to children as described in detail in Part II. The amount of insulin used to induce hypoglycaemia was 4 U per square meter body surface.

The results are summarized in Table XIV. They are in good agreement with those published in the excellent survey of Greenwood et al. (1966).

TABLE XIII. *Fasting levels of HGH in normal children and adults*

Source	No. of patients	Mean ± S.D. HGH ng/ml	Range HGH ng/ml
Age 1— 5 years	10	5.2±3.4	<1—12
Age 6—10 years	10	4.4±3.9	<1—11
Age 11—15 years	10	4.9±3.3	<1—10
Adults	10	1.2±1.0	<1—11

TABLE XIV. *HGH increase and glucose decrease to insulin-induced hypoglycaemia in adults*

HGH maximal increase. Mean ±S.D.	31.2±13.7 ng/ml
HGH maximal increase. Range	11 —62 ng/ml
Blood glucose minimal level. Mean ±S.D.	29.8± 6.2 mg/100 ml
Blood glucose minimal level. Range	21 —34 mg/100 ml

CHAPTER VII

Use of the labeled ^{131}I HGH for detection of antibodies

The labeled antigen is very useful for the purpose of detecting antibodies. If the labeled antigen when incubated with serum moves from its characteristic position in the free state to the position that in the separating system is occupied by bound antigen, then that serum will contain specific antibodies.

For growth hormone this is of special interest since it has been published that treatment with HGH will give rise to antibodies against HGH in some patients (Széky et al. 1962, Roth et al. 1964).

Method for detection of HGH-antibodies

The serum to be tested was serially diluted 1:1, 1:2, 1:4, 1:16, 1:32, 1:64 etc. with buffer 3. From each dilution step 500 µl was incubated with 0.4 ng ^{131}I HGH for 24 hours at +4°C. The separation was performed with the buffer-flow technique. The highest dilution that bound 50 % of the labeled hormone was taken as titre.

Results

All patients treated with HGH in our hands have been repeatedly tested for the presence of antibodies but none have been detected. In two serum samples with earlier proved antibodies (gift from Prof. A. Prader, Zürich, Switzerland) the antibodies have been verified. These two test samples were always run as controls when tests for the presence of antibodies were performed. The only explanation for that is that our patients so far have not produced antibodies. This will be discussed further in Part II.

CHAPTER VIII

General discussion

In the radioimmunoassay it is assumed that the radioactively labeled and unlabeled hormones behave identically and that the circulating hormone is chemically and immunologically identical with the standard hormone preparation.

The growth hormone used for this study has been prepared by the method of Roos et al. (1963). The method of preparation is rather mild, all procedures being performed in solutions of nearly neutral pH, and the method is favored by many investigators since the product seems less heterogeneous than most other preparations (Donato et al. 1966). This growth hormone, however, can be separated into several fractions as can other available preparations (Raben 1959, Ferguson and Wallace 1961, Gumbach and Kaplan 1962, Roos et al. 1963 and Hunter 1965). In most instances such fractions have been shown to possess identical antigenic and/or biological activity (Gumbach and Kaplan 1962). In some, however, quantitative differences have been shown (Ferguson and Wallace 1963, Hunter 1965). For the growth hormone used in this study it has been shown that one of four fractions, a minor portion of the total hormone preparation, has a decreased immunological activity but identical biological activity when compared with the other fractions. Furthermore Trygstad (1967) has been able to separate a lipid-mobilizing factor from the crude human pituitary extract and the subsequent separation of growth hormone yielded a more homogeneous somatotrophic hormone which gave a somatotrophic activity that was about 25 % higher on a weight basis than the HGH prepared according to the method of Roos et al. (1963). That would agree with the suggestion by Levine and Luft (1964) that what we call HGH is a combination of two hormones: one a growth promoting and the other a metabolic hormone. Available HGH would probably contain these two hormones in varying proportions.

Since radioimmunoassay is an inhibition technique and since plasma inhibits the reaction between labeled HGH and its antiserum, the plasma contains a protein which is immunologically identical with standard HGH obtained from human pituitaries if there is an exact parallel between equal dilutions of standard HGH and plasma. That is shown for this HGH preparation in Fig. 20 and has been shown for other preparations (Hunter and Greenwood 1964). Until the growth hormone from plasma is quantitatively isolated and characterised it cannot be proved if its biological activity is similar to the immunological activity, and if the immunological activity measured is representative for the metabolic and/or the somatotrophic action of HGH. Thus the HGH concentration in plasma obtained with radioimmunoassay should be called immunoreactive growth hormone. The results published by different investigators however do not differ greatly and so far the method studied above is no exception. In the near future an international standard will be presented by W.H.O. This will make it easier to compare results obtained in different laboratories.

The preparation of labeled growth hormone and antiserum used for the method described above is similar to those used by other authors. The labeled hormone seems to be comparable with those used in other methods. The antiserum shows a sufficient avidity to the antigen giving a slope of the

standard curve (Fig. 5) of about 8 %. (The slope is defined as the decrease in per cent bound per doubling dose of standard (Hunter 1967 a). The steeper the slope of the standard curve, the greater the discrimination between two unknown samples and the greater the precision). The slope is sufficient to give good precision but not so steep that the working range is restricted.

The problem of damaged HGH has been neglected in many radioimmunoassay systems. Berson et al. (1964) have stated that the sensitivity and precision of immunoassays is dependent upon the amount of damaged hormone present. For the present method it is shown that an increase in incubation damage will diminish the slope of the standard curve, and that will decrease the precision, with a corresponding increase in S.D. (Table III and Fig. 18).

Damaged HGH as shown by appearance of immunologically inert material may be caused by many factors. 1. Preparation of the hormone. Some studies indicate that HGH preparations prepared from the same starting material but handled in different ways during preparation show varying amounts of damage when labeled simultaneously under the same conditions (Westphal et al. 1968). 2. Manipulation with the hormone. As stated in the study above freezing and lyophylizing of the hormone increased the amount of damage. 3. Iodination of the hormone. a) Alteration of the hormone molecule due to substitution of iodine. If peptide hormones are heavily labeled there seem to be such alterations in the molecule that its immunological and chemical behaviour is altered. For HGH Hunter (1967 a) has shown that such alterations are not detectable if 7 or less atoms of iodine are substituted on the molecule. The labeling procedure used in this study will give less than 1 atom of iodine per molecule of protein. b) Radiation damage. This has been minimized in many ways. All reagents are kept as concentrated as possible, the total amount of ^{131}Iodide (2 mc) is small and the time of preparation very short. The separation on Sephadex is fast and after preparation the ^{131}I HGH is protected by the addition of protein and diluted. c) Chemical damage due to reagents used for labeling. Greenwood et al. (1963) could not prove any damage caused by chloramine-T when used in their labeling technique. Later Glover et al. (1967) stated that some damage of the protein may be due to the use of chloramine-T. The labeling method used in this investigation should be studied to see if a decrease in the amount of chloramine-T and consequently in meta-bisulfite will decrease the amount of damage without greater decrease in the yield of the labeling procedure resulting in decrease of the specific activity. d) Chemical damage due to noxious impurities in the radiochemical reagent. Such must occur at irregular intervals. As stated before, in periods the labeling has given a labeled product with a high amount of damage and a low yield of the labeling procedure. The results were identical for insulin when insulin and HGH were labeled simultaneously in this laboratory. The same experience has been claimed by Yalow and Berson (1967) and Hunter (1967 b). In 1963 Hunter was able to label HGH with specific activities up to 600 μc/μg using 4 mc ^{131}Iodide for 5 μg HGH without detectable damage. Since 1964, however, damage frequently has occured when 2 mc were used for 5 μg HGH. It is an urgent desire of all concerned with labeling of proteins with these radioisotopes that the commercial producers of the isotope solve this problem. 4. Incubation with plasma. This problem has been discussed in detail for insulin by Yalow and Berson (1964). Incubation of ^{131}I HGH with diluted plasma (1:5 or more) will give only minor interference but the longer the incubation time the more pronounced is the problem. For this method it is shown in Fig. 16 that the increase of S.D. in the 6 days incubated samples is caused by increasing damage. The damage is caused by the protein and is due to alteration in the

physio-chemical and immunological properties of the labeled antigen. The potency to cause damage seems to vary between different plasmas. It is not prevented by the enzyme inhibitor difluorophosphate (Roth et al. 1964).

A factor not previously studied is the influence of hemolysis. As shown in Table X a moderate hemolysis can be tolerated, while a pronounced hemolysis increases the incubation damage as it is tested in this system. To the author's knowledge no studies of the influence of hemolysis in other immunoassay systems have been published, so it is not possible to decide whether the effect of hemolysis is general or affects only the buffer-flow chromatography separation used in this study.

In the immunoassay described above, as well as in systems using electrophoresis or chromato-electrophoresis for separation of free and antibody-bound hormones, the damaged hormone will move with the antibody bound. If no correction for the damage is performed it will result in an underestimation. Thus for the standard curve as well as for each separate plasma estimated the approximate total amount of damage has been calculated as has been described earlier. An upper limit of damage has been stated to be about 35 %.

Of other separating systems, the double-antibody technique is the most frequently used (Utiger & Parker 1962, Hartog et al. 1964 a, Schalch and Parker 1964, Cerasi et al. 1966, and Root et al. 1967). The interference of damage has not been discussed by the authors but should result in an overestimation since the immunologically inert damaged components are held in solution and read as free antigen. Some other factors however will influence the results in the opposite direction. Morgan et al. (1964) showed a heat labile plasma inhibitor and that the method was influenced by the total amount of protein. Cerasi et al. (1966) demonstrated that different HGH levels could be obtained when the amount of antiserum or the incubation time was changed. So the double-antibody system causes several difficulties that make comparison between HGH levels obtained via different separation methods delicate.

Storage of blood plasma samples. The method used has been carefully tested especially for its application on neonatal plasma. However, no differences between adolescent and neonatal plasma with regard to their behaviour in the assay have been proved with one exception. When plasma or blood is kept at room temperature the HGH levels obtained successively decrease. For adolescent plasma no alteration is noted within 24 hours, a fact which would permit transport of plasma without freezing or other precautions from a wide area surrounding the laboratory. For neonatal plasma however a significant decrease can be noted after 14 hours. As for whole blood a significant decrease will be detectable after 4 hours for adolescent blood and within 2 hours for neonatal. The storage at room temperature is not followed by any increase in incubation damage. The reason for the decreased HGH levels measured may be due to changes in the immunological activity induced by enzymes in plasma and blood. Experiments with enzyme inhibitors however have not been performed. Consumption of growth hormone by the corpuscular elements of the blood is another possibility. However storage of plasma at —20°C will completely inhibit alterations in plasma since estimations of identical plasma over a time interval of two years gave identical results. Neither does freezing and thawing plasma give any changes in HGH levels obtained.

CHAPTER IX

Final conclusions

The radioimmuno-chromatographic assay for HGH described seems suitable for the study of immunoreactive growth hormone in neonatal and adult plasma. The precision and sensivity is comparable with other assays published, and the results obtained agree with those published by others. The problem of degradation damage has to be given special attention. However, it is simple to make adequate corrections. The incubation time of four days permits a convenient laboratory routine and the capacity is sufficient for most purposes. The prepared labeled ^{131}I HGH and the flow chromatography separation are suitable for detection of antibody against HGH which is of importance for patients treated with HGH.

PART II

Clinical Study of Human Growth Hormone in Children and Newborn Infants

CHAPTER X

Introduction

Since the radioimmunoassay for growth hormone became available in 1963 our knowledge of the regulation and function of growth hormone has rapidly increased. The first investigations (Hunter and Greenwood, 1964) showed rather low HGH levels in infants as well as in adults, the differences between patients were fairly large and normal control subjects showed resting levels of less than 1 ng/ml, which was the lowest sensitivity in routine assay for most methods. Roth et al. (1963 b) were the first to show that the HGH level was influenced by the actual blood glucose level. Hypoglycaemia was followed by a marked increase in plasma HGH if induced by insulin or by infusion of fructose or by 2-desoxy-D glucose. Hyperglycaemia following glucose administration decreased growth hormone levels, but the subsequent decline of blood sugar concentration was followed by an increase in growth hormone. This made it possible to suggest tests for growth hormone release. A standardization of a test for the hypothalamo-pituitary axis and the secretion of growth hormone has been studied and proposed by Greenwood et al. (1966).

In the present study the HGH levels during the intravenous insulin tolerance test have been studied in four groups of patients.

1. Normal children.
2. Children with retardation of growth.
3. Children during long-term treatment with corticosteroids.
4. Normal infants, infants of diabetic mothers and premature infants during the first week of life.

The growth hormone estimations have been performed by the method described in Part I. Thus the HGH levels obtained represent immunoreactive growth hormone.

In addition, the results of treatment with HGH prepared by the method of Roos et al. (1963) will be presented.

CHAPTER XI

Growth hormone release during insulin tolerance test in normal children

The first study of growth hormone levels in normal children was published by Hunter and Greenwood (1964). They showed that fasting levels in children were somewhat higher than thos in adults but even in normal children fasting plasma samples may contain less than 1 ng/ml of HGH. Several authors (Roth et al. 1963 b, Frantz and Rabkin 1964 and Greenwood et al., 1966) have published data about HGH response to insulin induced hypoglycaemia in adults but until recently only small series are published about similar studies in normal children (Greenwood 1964, Root et al. 1967, Parker et al. 1967 and Kaplan et al. 1968). The purpose of the present investigation was to study the HGH release due to insulin-induced hypoglycaemia in normal children.

Material and methods

Subjects. Sixteen children aged 5 to 15 years with normal growth and without any suspicion of endocrine disease were studied. They were all admitted to the hospital and were accustomed to the hospital routine.

Insulin tolerance test. Prior to the study the children fasted at least 12 hours. The test was started at about 8 o'clock in the morning. A scalp vein needle was put in a peripheral vein and a slow saline infusion was started and maintained throughout the test to prevent clotting and make an immediate infusion of glucose possible. The total amount of saline infused did not exceed 100 ml. Before a blood sample was drawn the first ml containing blood and saline was discarded.

Crystalline insulin (Vitrum, Stockholm, Sweden) 4 U per square meter body surface was diluted to 1 ml in 0.9 % saline and injected I.V. followed by a wash of about 5 ml physiological saline. The insulin amount used in this study corresponds to about 1.5 U per kg body weight. Blood samples were withdrawn prior to, and 15, 30, 45, 60, 75 90 and 120 minutes after, the injection of the insulin. The blood was put in glass tubes containing a small amount of heparin (Vitrum, Stockholm, Sweden). Blood for glucose estimation in duplicate was withdrawn immediately and precipitated. The glass tubes were placed in a beaker containing pieces of ice and when all samples were taken, the blood was spun down, the plasma withdrawn and frozen.

Blood glucose determinations were performed by an enzymatic method using 0.025 M NaOH and 10 % $ZnSO_4$—$7H_2O$ as precipitating agents (Hjelm and de Verdier 1963).

Growth hormone was estimated by the method described in Part I. All estimations were made in triplicate. Control samples for the estimation of incubation damage were taken from the first and the last tube of every load and the mean was used for correction of the bound fraction (page 13).

Results

The results are summarized in Table XV.

The blood glucose decrease was sufficient, the lowest levels were less than 50 % of fasting levels. In most of the children a slight sweating 25—30 minutes after the insulin gift was noted and few complained about moderate dizziness but is was not necessary to interrupt the test in any patient.

The mean ± S.D. of the fasting *level of HGH* was 4.1 ± 2.4 ng/ml with a range between <1 (in two patients) to 8 ng/ml. The peak level in most patients (10/16) was obtained 45 minutes after the insulin gift. Four patients reached their peak levels 30 minutes after the insulin injection and two after 60 minutes. The mean increment ± S.D. from the fasting level was 29.1 ± 11.5 ng/ml. The highest increments occurred in the youngest children. The response fell with increasing age until a minimum was reached at the age of 11 years. Then the response increased again (Fig. 23).

Fig. 23. Growth hormone increments above fasting levels following insulin-induced hypoglycaemia in 16 normal children aged 5—15 years.

Discussion

The fasting levels of HGH for the age group studied corresponds well with those published by Greenwood et al. (1964) and are slightly higher than those published by Root et al. (1967) and Kaplan et al. (1968).

The HGH response to hypoglycaemia in children is comparable with the response now found in adults (page 30) and in agreement with the results found in children by Greenwood (1964) using an electrophoretic technique. The results of Parker et al. (1967) are comparable with the present results since no differences were shown between children and adults. However, their HGH levels are slightly lower than the present results and those of Greenwood et al. (1966). This is probably explained by Parker's double antibody technique. A lower response in children is reported by Root et al. (1967) using a double antibody technique and by Kaplan et al. (1968) using a chromato-electrophoretic technique. However, no parallel studies of children and adults have been published by any of these authors. Several reasons for this difference between the studies are possible. There are variations in the immunoassay techniques as well as in HGH standards since no international standard is available at this time. Another factor not studied in detail is the effect of different amounts of insulin used to induce hypoglycaemia. In the excellent survey of HGH response to insulin-induced hypoglycaemia in adults by Greenwood et al. (1966) it was shown that the HGH response varied with the amount of insulin. Most reliable results were obtained with an insulin amount of 0.15 U per kg body weight. In the present study the amount of insulin (4 U/m^2) corresponds to about 0.15 U per kg body weight. In all other studies on children mentioned above, however, 0.1 U/kg was used. The suggestion by Kaplan et al. (1968) that the reason for the depressed response in HGH to hypoglycaemia in children is due to an altered sensitivity of the hypothalamus could be supported by the present study. The greater amount of insulin used in this study induced a more powerful stimulus to the hypothalamus resulting in a pronounced HGH release.

In the present study the HGH response seems to change with the age of the patient. The highest response is obtained in the ranges 5—7 and 13—15 years, periods of most rapid growth. The same age variations have been found by Stimmler and Brown (1967) and

TABLE XV. *Growth hormone and blood glucose levels during IV insulin tolerance test in 16 normal children aged 5—15 years*

Case	Age, Years / Months	Sex	Time in minutes HGH in ng/ml Blood glucose in mg/100 ml							
			0	15	30	45	60	75	90	120
1	5/1	f	4 56	6 34	41 21	62 32	39 48	17 52	10 60	2 68
2	6/2	m	4.5 72	7 38	38 36	44 44	31 58	20 75	12 76	<1 69
3	7/3	f	1.5 64	3.5 40	38 30	30 38	24 51	21 65	10 72	7 78
4	7/6	m	<1 82	7 41	44 38	38 42	24 54	18 70	8 75	<1 72
5	8/4	m	1.5 67	7 40	18 32	44 42	40 57	18 65	16 71	6 67
6	9/4	f	2.5 72	1.5 37	14 30	12 48	10 59	5 69	7 77	3 65
7	9/4	m	5.5 54	5 31	10 23	28 36	32 49	30 55	21 62	5 65
8	10/3	f	3.5 60	5 38	12 28	21 37	18 51	12 59	8 71	2.5 68
9	10/7	m	6 73	7 40	25 24	20 38	18 48	15 55	5 75	<1 88
10	11/0	f	<1 68	2 41	10 31	18 42	24 57	16 68	10 73	8 92
11	11/3	m	3 78	6 37	10 30	17 47	13 61	10 65	8 76	8 63
12	12/1	f	4 55	6 36	12 23	27 38	26 51	19 60	12 71	10 68
13	12/8	f	5.5 72	8 39	18 31	38 44	29 57	21 62	10 71	12 67
14	13/2	m	2 88	10 51	32 39	43 49	35 57	18 68	<1 79	1.5 92
15	14/9	f	7 63	9 38	17 29	28 41	20 55	14 68	8 71	1 65
16	15/6	m	4 59	5 31	24 24	32 38	29 51	22 59	10 71	8 63

Kaplan et al. (1968) in their studies on children of short stature.

The range in HGH response is very wide for all studies presented. This makes it difficult to establish the lowest response that can be accepted as normal when the method is used for the comparison between normal subjects and those with various diseases, especially growth retardation. For the present study a growth hormone increase above 10 ng/ml has been adopted as lower limit. This is in agreement with the studies of Greenwood et al. (1966) and Frohman et al. (1967), and comparable with the results of Kaplan et al. (1968). Borderline response occurs in some cases and further provocation tests may be necessary to prove the right diagnosis, as will be discussed later (Chapter XII).

Summary

The growth hormone response to insulin-induced hypoglycaemia has been studied in 16 children aged 5 to 15 years. The mean ± S.D. HGH increase during hypoglycaemia was 29.1 ± 11.5 ng/ml which is of approximately the same size as earlier found in adults. The response varied with age of the children, the response being most pronounced in the age groups where growth is accelerated.

The results are in agreement with those published by Greenwood (1964) and Parker et al. (1967) but not in agreement with those published by Root et al. (1967) and Kaplan et al. (1968). Differences in HGH standard, immunoassay techniques and amount of insulin used to induce hypoglycaemia are probable explanations for the differences. It is concluded from the results of the present study that a normal response to insulin-induced hypoglycaemia can be defined as an HGH increase of 10 ng/ml above fasting levels.

CHAPTER XII

Growth hormone release during insulin tolerance test on children of short stature

The diagnosis of hypopituitary dwarfism in children has been a matter of great difficulty. Stunted growth is the obligate symptom but since this is a common occurrence in childhood the differential diagnoses have made necessary a number of time demanding investigations which too often have not yielded a precise answer. Previously it was believed that the growth in hypopituitarism in children was not impaired during the first two years of life. However, in the review of Brasel et al. (1965) it was stated that in nearly half of the children with idiopathic hypopituitarism the growth was retarded at the age of two years and 35 % showed growth retardation already at the age of one year. This is in agreement with the results of Aarskog (1963). Absence of gross structural abnormalities such as craniopharyngioma and of deficiency in other trophic hormones measurable by functional defects in the target glands, make the diagnosis idiopathic hypopituitarism very difficult. An increased insulin sensitivity has been used to confirm the diagnosis hypopituitary dwarfism and the blood glucose levels during insulin-glucose tolerance test (Trygstad 1965) were the main diagnostic evidence for Seip and Trygstad (1966) to find patients suitable for HGH treatment.

Measurement of growth hormone levels in children of short stature became possible in 1963 and studies on patients with stunted growth have been published by Root et al. (1967), Parker et al. (1967), Frohman et al. (1967), Stimmler and Brown (1967) and Kaplan et al. (1968). In the present investigation children with suspected hypopituitarism, premordial dwarfism, constitutional growth retardation, Turner's syndrome and chondrodystrophy have been studied. The aim of the investigation was to study the HGH response to insulin-induced hypoglycaemia on children of short stature, and to see whether a subnormal response as defined in Chapter XI was correlated to hypopituitary dwarfism.

Material and methods

Subjects. Growth hormone response to insulin-induced hypoglycaemia has been studied in 31 children of short stature. Furthermore a boy of normal height with a craniopharyngioma was studied prior to and following hypophysectomy. The patients have been divided into four groups: 1) hypopituitary dwarfs, 2) premordial dwarfism and constitutional growth delay, 3) Turner's syndrome and 4) chondrodystrophic dwarfs. The classification was based on criteria stated by Wilkins (1965). Furthemore successful treatment with HGH (chapter XIII) was required for the diagnosis of hypopituitarism. However, treatment could not be tried in all children of short stature owing to the limited supply of HGH. Patients with subnormal or borderline HGH response to insulin-induced hypoglycaemia as defined in chapter XI have been treated for at least six months. Patients with no clinical signs of hypopituitarism and with normal HGH response have not been treated. Using this basis for classification the groups are as follows.

Hypopituitarism, nine children. In five children with idiopathic hypopituitarism, deficiencies in multiple trophic hormones were found; in two only a single growth hormone deficiency has been shown so far.

In two patients the hypopituitarism was due to craniopharyngioma, one of these was of normal height. HGH deficiency and diabetes insipidus, but without any tumour demonstrable by repeated pneumoencephalograms, were present in one child.

Clinical data pertaining to the patients are summarized in Table XVI.

Patients with constitutional growth delay and with premordial dwarfism. No distinction is made between these groups since each group probably represents a heterogeneous mixture with respect to etiology. Four of the fifteen patients showing borderline HGH response to insulin-induced hypoglycaemia and with clinical signs that made hypopituitary origin of the growth retardation probable have been treated for six months with HGH without success and are thus included in this group. Clinical data pertaining to these patients are summarized in Table XVII.

Five children with Turner's syndrome. All five children showed clinical stigmata of Turner's syndrome.

Two patients with chondrodystrophy. They showed characteristic clinical and radiological stigmata. Clinical data in Table XVIII.

Insulin tolerance test. The test, including measurements of blood glucose and HGH, was permormed as described earlier (page 39).

The thyrotrophic function was studied indirectly by measurement of PBI* using a Technicon Auto Analyzer (1964). The normal range in the laboratory is 3.5—7.5 µg/100 ml.

ACTH-function was studied indirectly by measurement of the output of 17-OH corticosteroids in urine on oral administration of 3 g metyrapone divided in 6 doses during 24 hours.

The gonadotrophic function was studied in 28 patients. The excretion of urinary FSH** was estimated after extraction according to Johnsen (1958) by the method of Steelman and Pohley (1953) modified using 40 U of HCG per animal, one injection per day for three days. The standard preparation used was the 2nd IRP for FSH. Levels less than 4 U/24 hours were undetectable. The LH*** excretion in urine has been estimated by the hemagglutination inhibition technique (Wide et al. 1961) in a minority of the patients and by the radioimmunosorbent technique (Wide and Porath, 1966) in most patients. The standard used was 2nd IRP for LH. Levels less than 4 U/24 hours indicate insignificant levels.

*The karyotype*** has been studied in all patients in cultures of leucocytes from peripheral blood.

Measurements of standing height. In all children height measurements were performed in the morning the first three days in the hospital, the mean was accepted as the "true" height.

Height age was calculated from the diagram of Karlberg-Iggbom (1959).

Bone age estimations were performed by the method of Eklöf and Ringertz (1967).

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*Kindly performed at the Department of Clinical Chemistry, University Hospital, Uppsala, Sweden, under the supervision of Ass. Prof. C.-H. de Verdier.

**Kindly performed by Ass. Prof. B. H. Persson, Department of Gynecology and Obstetrics, University Hospital, Uppsala, Sweden.

***Kindly performed by Ass. Prof. L. Wide, Department of Clinical Chemistry, University Hospital, Uppsala, Sweden.

****Kindly performed by Ass. Prof. K.-H. Gustafsson, Head of the Cytogenetic Laboratory of the Peadiatric Clinic, University Hospital, Uppsala, Sweden.

I want to express my thanks for the generous help with these investigations.

TABLE XVI. *Clinical data on children with hypopituitarism*

Case	Sex	Age, Years/ Months	Heigth, cm	Height age, Years/Months	Bone age, Years/Months	Birth weight, g	Gestation time, Weeks
1*	m	5/1	96	3/0	2/6	3600	40
2	m	6/7	97	3/2	4/0	3750	44
3	f	8/2	107	4/7	5/0	1850	35
4	m	15/5	117	6/6	8/0	3200	40
5	m	16/1	138	10/2	10/0	—	—
6	f	18/1	128	8/5	11/0	4000	41
7	m	25/2	135	9/10	10/0	3500	40
8**	f	8/11	114	6/0	7/6	4010	40
9***	m	11/0	126	7/11	9/6	3200	40
10***	m	11/3	150	12/6	12/0	3790	40

*Died in a neuroblastoma 5 months after investigation and before start of HGH treatment.
**Diabetes incipidus started three years prior to the investigation.
***Hypophysectomized for craniopharyngioma.

TABLE XVII. *Clinical data on children with premordial and constitutional dwarfism*

Case	Sex	Age, Years/Months	Height, cm	Height age, Years/Months	Bone age, Years/Months	Birth weight, g	Gestation time, Weeks
11	f	5/0	95	3/0	4/0	2680	42
12	m	5/8	103	4/0	5/0	2730	39
13	f	6/0	100	3/8	3/6	3900	40
14	m	6/7	104	4/5	6/6	3100	40
15	f	7/6	104	4/5	7/0	2400	38
16*	m	8/9	115	6/0	8/6	3050	40
17	m	9/0	102	3/11	4/0	2050	40
18**	m	10/4	100	3/8	8/6	1750	40
19	f	13/2	135	9/9	12/0	2700	39
20	f	13/4	111	5/4	11/0	3380	40
21	m	13/6	135	9/9	13/6	3150	40
22	m	13/7	135	9/9	10/0	3770	40
23	m	13/7	133	9/6	11/0	3160	40
24	m	15/2	124	7/10	13/0	2000	36
25	m	18/0	146	11/8	13/0	3200	40

*Bird headed dwarf
**"Male Turner"

TABLE XVIII. *Clinical data on children with Turner's syndrome and chondrodystrophic dwarfism*

Case	Sex	Age, Years/Months	Height, cm	Height age, Years/Months	Bone age, Years/Months	Birth weight, g	Gestation time, Weeks
26	f	5/0	97	3/2	4/0	2550	40
27	f	11/0	120	7/7	10/6	3420	42
28	f	13/1	135	9/9	13/0	3380	40
29	f	17/1	135	9/9	13/0	2800	40
30	f	21/8	144	11/4	15/0	2800	41
31	m	4/0	94	2/8	4/0	3250	40
32	m	10/1	118	6/9	10/0	3530	40

Results

The increments of HGH to insulin-induced hypoglycaemia are compared with the increments for normal children in Fig. 24.

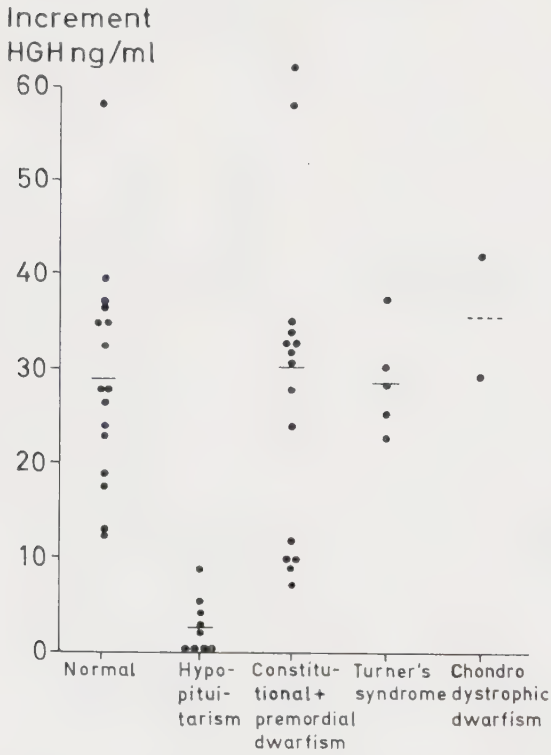

Fig. 24. Growth hormone increments from fasting levels following insulin-induced hypoglycaemia in 16 normal children and 31 children with stunted growth. The horisontal lines indicate the mean increment for the group.

Hypopituitarism. Blood glucose and HGH levels are shown in Table XIX. Five of the patients showed a pathological insulin load with low blood glucose levels and persistent hypoglycaemia. In one case (no. 2) the insulin load had to be interrupted by glucose infusion 28 minutes after the insulin gift. Two patients had a history of spontaneous hypoglycaemia.

Base levels of HGH were less than 1 ng/ml in six of the patients. Four patients showed no measurable increase in HGH levels during the insulin load, and four patients showed subnormal response. Furthermore one patient with stunted growth due to craniopharyngioma showed a subnormal increase and one patient with normal growth in spite of craniopharyngioma showed normal HGH response prior to the operation but failed to increase in HGH after the hypophysectomy.

The results of other laboratory investigations are summarized in Table XX.

PBI was decreased and the response to metyrapone was poor in 5 of the 7 patients with idiopathic hypopituitarism. The patients with craniopharyngioma had normal response. Only one patient (one with craniopharyngioma) showed any gonadotrophic activity. All patients had a normal karyotype. Height age and bone age were markedly retarded in all patients with HGH deficiency at the time of the study. In three of the patients it was possible to detect growth retardation at the age of two years. As the years passed the gap between the child's growth and normal growth increased for all patients.

Constitutional and premordial dwarfism. The laboratory investigations are summarized in Table XXI. The blood glucose was decreased to less than 50 % of the fasting level in all children with a mean of 41 % of fasting level and a range of 28—47 %. Two patients showed an increased insulin sentitivity with prolonged hypoglycaemia.

Fasting levels of HGH varied between 1 and 8 ng/ml and the maximal increment between 7 and 62 ng/ml with a mean $\pm$ S.D. of 30.9 $\pm$ 15.9 ng/ml. This is in agreement with the results obtained with normal children. Two patients showed insufficient HGH increase, defined earlier as an increment less than 10 ng/ml (page 42) and these two patients also showed an increased insulin sensitivity. One of them (case 22) had a history of hypoglycaemic weakness. Two patients showed borderline HGH response.

PBI was normal in all children. A poor response to metyrapone was noticed in three patients of which two were the patients with subnormal HGH response and one showed a borderline HGH response. The gonadotro-

TABLE XIX. *Growth hormone and blood glucose levels during IV insulin tolerance test in children with hypopituitarism*

Case	Time in minutes HGH in ng/ml Blood glucose in mg/100 ml							
	0	15	30	45	60	75	90	120
1	2.0 67	2.5 35	4.0 28	5.0 21	3.0 35	<1 40	<1 42	1.5 40
2	<1 69	<1 26	<1 11	<1 310*				
3	1.5 79	1.0 55	2.0 25	2.5 28	3.5 37	3.0 45	3.5 50	<1 55**
4	<1 55	<1 32	<1 18	<1 25	<1 38	<1 44	<1 40	<1 75
5	<1 78	<1 34	3.0 28	5.5 32	4.0 33	3.5 44	<1 48	<1 52
6	<1 58	<1 28	<1 23	<1 22	<1 28	<1 32	<1 37	<1 41
7	<1 68	<1 22	<1 30	<1 20	<1 35	<1 39	<1 47	<1 58
8	1.5 78	1.5 34	4.0 28	5.5 32	5.0 48	4.0 69	1.5 77	2.0 75
9	<1 70	1.5 32	4.0 32	4.0 57	6.0 65	9.0 63	8.5 68	6.0 70
10 a	5.5 68	6.5 32	18 30	34 42	28 58	21 72	8.0 78	4.0 75
10 b	<1 77	<1 28	<1 30	<1 38	<1 51	<1 72	<1 82	<1 78

10 a prior to operation of craniopharyngioma.
10 b 3 months after operation.
*After the injection of glucose.
**5 hours after insulin injection the blood glucose was 36 mg/100 ml, in spite of two meals in the meantime.

phins were moderately elevated in 5 of 7 patients aged 11 years or more. Of the two patients without gonadotrophic activity one showed a subnormal HGH response, and the other, a borderline response.

The karyotype was normal in all children. Height age was retarded with a minimum of two years. Bone age was retarded in ten of the patients.

In all patients with Turner's syndrome (laboratory results in Table XXII) the blood glucose fell to less than 50 % of fasting level (mean 40 %, range 31—45 %).

Fasting levels of HGH varied between 3 and 8 ng/ml and the response to hypoglycaemia was of the same order as that for normal children. The mean ± S.D. was 28.4 ± 5.7 ng/ml and the range 22—37 ng/ml.

The PBI and the response to metyrapone was normal. The gonadotrophins were high in patients aged 13 years or more. In four patients the karyotype was 44 + XO and in

one patient the karyotype was 44 + XX_L with iso-chromosome for the long arm of the X chromosome. The bone age was moderately delayed in the patients aged 17 and 21 years.

The two patients with chondodystrophic dwarfism (laboratory results in Table XXII) showed a normal blood glucose decrease. The fasting levels of HGH were 3 and 6 ng/ml, respectively and the increments 42 and 29 ng/ml, respectively. The karyotype was normal as was the bone age.

Discussion

Hypopituitarism. Five of the patients showed an increased sensitivity to insulin. These five children had a poor response to metyrapone so a combination of HGH and ACTH deficiency seems to increase the risks for serious hypoglycaemia. This is in agreement with the results of Kaplan et al. (1968). The blood glucose has not been studied in the period between 20 and 30 minutes after insulin injection which makes a comparison with the study of Trygstad (1965) impossible.

All patients showed low HGH response. This is in agreement with all earlier studies. Five patients with idiopathic hypopituitarism showed a deficiency in corticotrophic as well as in thyrotrophic action.

The retardation of bone age was most pronounced in this group of patients when compared to other groups of stunted growth as stated earlier by Clayton et al. (1967).

Constitutional retarded growth and premordial dwarfism. The reason for the increased insulin sensitivity in two cases (nos. 22 and 25) combined with spontaneous hypoglycaemia in one of them is not clearly understood. Both of them showed a subnormal HGH response, a poor response to metyrapone and a marked retardation of both bone age and height age. This made the diagnosis hypopituitary dwarfism probable and the patients were treated for six months with 2 mg HGH twice a week. During this period, however, no increase in growth occurred. Two other patients showed borderline response to HGH (cases 17 and 23) one of them showing a poor metyrapone response (23) and both having markedly retarded bone age and height age. Even in these patients treatment with HGH had been tried without any acceleration in growth. The discovery of patients with stunted growth of non-pituitary origin but poor response to insulin-induced hypoglycaemia is in agreement with the results of Kaplan et al. (1965), Parker et al. (1967), Stimmler and Brown (1967) and Kaplan et al. (1968).

PBI was normal in all patients and the response to metyrapone was normal in all except those discussed above. Gonadotrophins were present in all except two patients aged 13 years or more. Absence of gonadotrophins was noted in cases number 22 and 23 which increased the suspicion of pituitary origin of growth retardation in these patients. To the author's knowledge no studies of gonadotrophins in these groups of patients have been published.

The retardation of bone age varies from patient to patient since the group is rather heterogeneous. However, the bone age is more retarded in those patients which may be classified as premordial dwarfs. The growth for the constitutional dwarfs seemed to follow normal growth, the gap between actual height and normal height was almost constant during the years the patients have been followed. As for most of the premordial dwarfs the gap between actual height and normal height seemed to increase but not to the same extent as in hypopituitarism. This was also found by Clayton et al. (1967).

Turner's syndrome and chondrodystrophic dwarfism. The results for the patients with Turner's syndrome are in agreement with the results of Root et el. (1967), and Kaplan et al. (1968). As for the two patients with chondrodystrophy the growth hormone fasting levels and the release was normal. This is in agreement with results found by Beck (1965).

TABLE XX. *Laboratory investigations on children with hypopituitarism*

Case	PBI, µg/100 ml	Metyrapone response	Gonadotrophins, Units/24 hours FSH	LH
1	8.1	normal	—	—
2	2.0	poor	—	—
3	3.6	poor	<4	<4
4	2.7	poor	<4	<4
5	4.7	normal	<4	<4
6	3.0	poor	<4	<2
7	3.2	poor	<4	<4
8	5.4	normal	<4	<2
9	4.5	normal	<4	<4
10	5.8	normal	<4	6

TABLE XXI. *Laboratory investigations including HGH increments from fasting levels following IV insulin tolerance test in children with premordial and constitutional dwarfism*

Case	PBI, µg/100 ml	Metyrapone response	Gonadotrophins, Units/24 hours FSH	LH	Insulin load	HGH ng/ml, Fasting level	Increment
11	7.2	normal	<4	<4	normal	8	34
12	7.0	normal	<4	<4	normal	3	33
13	8.2	normal	<4	<2	normal	2.5	12
14	6.9	normal	<4	<4	normal	2	28
15	7.3	normal	<4	<2	normal	7	35
16	6.0	normal	<4	<4	normal	4	33
17	7.8	normal	<4	<4	normal	<1	10
18	5.7	normal	<4	<4	normal	6	58
19	6.2	normal	4	8	normal	2	32
20	5.8	normal	4	8	normal	<1	24
21	5.5	normal	6	11	normal	5	31
22	5.7	poor	<4	<4	pathological	2	9
23	6.7	poor	<4	<4	normal	<1	10
24	4.4	normal	6	8	normal	6	62
25	4.3	poor	6	8	pathological	1.5	7

TABLE XXII. *Laboratory investigations including HGH increments from fasting levels following IV insulin tolerance test in children with Turner's syndrome and chondrodystrophic dwarfism*

Case	PBI, µg/100 ml	Metyrapone response	Gonadotrophins, Units/24 hours FSH	LH	Insulin load	HGH ng/ml, Fasting level	Ingrement
26	7.5	normal	<4	<4	normal	3.0	37
27	6.3	normal	4	4	normal	6.0	25
28	7.2	normal	100	117	normal	6.0	28
29	5.0	normal	50	40	normal	7.0	22
30	5.2	normal	80	107	normal	8.0	30
31	—	—	—	—	—	3.0	42
32	—	—	—	—	—	6.0	29

The response to insulin-induced hypoglycaemia was less than normal in all patients with dwarfism of pituitary origin. However, subnormal response was also shown in two patients where HGH deficiency was not the reason for the growth retardation. One factor that influences the HGH response seems to be fasting HGH concentration. Frohman et al. (1967) and Kaplan et al. (1968) have shown that subnormal response to hypoglycaemia is more common in patients where the fasting levels are high. This is not seen in the present study but its absence is probably coincidental, and the two patients with subnormal response in this study had low fasting levels of HGH. Hypothyroidism was found to decrease HGH response in three patients studied by Root et al. (1967). As shown by Hartog et al. (1964 b) and in a study by the author (chapter XIV) glucocorticoid excess also depresses HGH response. However, there were no signs of hypothyreosis or increased corticosteroid secretion in the two patients with subnormal response.

The response in HGH release to insulin-induced hypoglycaemia is a very useful test in the diagnosis of hyposomatotrophinism in most cases. The risk of severe hypoglycaemia can be neglected if adequate safeguards, including the immediate availability of glucose for intravenous injection, are arranged. In most cases the test will yield the correct diagnosis, but since borderline response occurs a number of further tests have been suggested.

The infusion of arginine causes a release of HGH as shown by Knopf et al. (1965). Comparative studies between the HGH release to insulin-induced hypoglycaemia and to arginine infusion have been published by Parker et al. (1967) and Frohman et al. (1967). The conclusion in both studies is that the arginine provocative test is a complement in the diagnosis of hyposomatotrophinism but even this test is not able to make an exact distinction in every single case. The advantage with the test is that there is no risk for hypoglycaemia.

The effect of vasopressin on HGH levels was studied by Gagliardino et al. (1967). In 15 normal subjects aged four months to 88 years an increase in HGH was measurable 30 to 60 minutes after an intramuscular injection of vasopressin. In four hypopituitary patients there was no difference between HGH release to insulin or vasopressin. The advantage of this test would be a single provocative test for HGH, ACTH (Gwinup 1965) and FSH (Dreyfus et al. 1963). However, at this time the knowledge about the vasopressin test for HGH release is too small to make it useful as a routine test.

Hyperglycaemia was early shown to induce a decrease in HGH levels, followed by an increase in consequence of blood sugar decrease (Roth 1963 b). This has been studied in detail by Hunter et al. (1967) who have described a simple screening test for growth hormone deficiency. The increased HGH levels are studied in 2—3 blood samples taken $2^1/_2$ to 4 hours after an oral glucose load of 1.4 g per kg. In patients with probable hypopituitarism they found agreement between this test and HGH response to insulin-induced hypoglycaemia. The advantage of a glucose test is that it is less unpleasant for the patient and that it probably is a more physiological stimulus.

The metabolic HGH test has been used by some investigators. Prader et al. (1964) showed that daily injections of 2 mg/m^2 of HGH increased the nitrogen retention during the 2nd and 6th day of treatment to a much greater extent in cases of pituitary dwarfism than in cases involving other causes of growth retardation. Hubble (1966) showed that the withdrawal of HGH after a test period of 5—9 days induced an increase in nitrogen excretion in non-hypopituitary dwarfs while hypopituitary children still retained nitrogen. These metabolic tests, however, are very time demanding and require very good cooperation of the patients since nothing seems to be more difficult in practice than an accurate collection of urine.

The insulin susceptibility has long been used as a diagnostic test. However, in this study as well as in others the insulin sensitivity seems to be marked only in patients where an ACTH deficiency is also present. Furthermore insulin sensitivity may be present without any hyposomatotrophinism.

The tests of other pituitary trophic hormones are valuable in some cases but they are all time demanding and do not prove HGH deficiency.

At this time no method for the study of HGH deficiency seems to be absolute reliable. There are patients on whom available diagnostic tests fail. In such cases a test period with HGH treatment seems to be the only possible way to make a clear diagnosis. It is necessary that this treatment is long enough since short treatment may be misleading due to seasonal changes in growth or due to transient acceleration of growth.

Summary

The HGH response to insulin-induced hypoglycaemia has been studied in 31 children with short stature. Ten patients have been classified as hypopituitary dwarfs, and all these patents showed an HGH increment of less than 10 ng/ml from fasting levels. In six of these there were signs of deficiency of other pituitary hormones and two had a craniopharyngioma. In fifteen children the diagnosis was premordial dwarfism or constitutional delay in growth. Two patients in this group showed a subnormal HGH response to insulin-induced hypoglycaemia. In five patients with Turner's syndrome and in two patients with chondrodystrophy the HGH response was normal when compared to a control group. Other parameters compared were the blood glucose levels during the insulin-induced hypoglycaemia, PBI, excretion of 17-OH corticosteroids after metyrapone, urinary gonadotrophins and the bone age. The difficulties in the interpretation of different tests are discussed and some other possible tests for the study of HGH release have been described. On the basis of this study measurements of HGH levels during insulin-induced hypoglycaemia is a useful aid in the diagnosis of hypopituitarism. When borderline results are found further tests or short term therapy with HGH is necessary.

CHAPTER XIII

Treatment of pituitary dwarfism with human growth hormone

in collaboration with Paul Roos*

The first successful treatment of a hypopituitary dwarf with human growth hormone (HGH) was reported by Raben (1958). During the following years several authors (Shepard et al. 1960, Vest et al. 1960, Escamilla et al. 1961, Aarskog 1963, Trafford et al. 1963, Soyka et al. 1964, Rosenbloom 1966, Seip and Trygstad 1966, Tanner and Whitehouse 1967, Prader et al. 1967 and Kaplan et al. 1968) have published their results on HGH therapy using various preparations. The general experience of HGH treatment, however, is limited due to lack of sufficient amounts of the hormone.

Only hypopituitary dwarfs have been proved to enhance their growth to HGH. No side effects to treatment with HGH have been reported but a limitation in the treatment is the development of antibodies to HGH. This was first shown for the Raben preparation by Széky et al. (1962). In the present investigation growth acceleration to therapy with HGH according to Roos et al. (1963) has been studied. Tests for the presence of antibodies have been performed in all patients with HGH therapy of 6 months or more.

Material and methods

Subjects. Nine patients with hypopituitary dwarfism (clinical data in Tables XVI and XX pages 45 and 49) have been treated. Furthermore four patients with doubtful hypopituitarism (cases number 17, 22, 23, 25 in Tables XVII and XXI pages 45 and 49) have been treated for 6 months. The clinical evaluation of the diagnosis has been described in Chapter XII.

Human growth hormone was prepared by Roos according to the method of Roos et al. (1963). The preparation was stored lyophylised and was dissolved prior to the intramuscular injection in 1 ml 0.9 % saline. The dose was for all patients 2 mg twice a week. Lack of hormone made interruptions in the treatment necessary.

Height was measured every third month during treatment and, if possible, at the same time of the day on all occasions.

HGH antibodies were determined according to the method described in Part I (Chapter VII). Blood samples were taken every six months even if an occasional interruption in the treatment had occurred. All patients were tested for the presence of antibodies in January, 1968.

Results

Hypopituitarism. The results are summarized in Table XXIII and shown for case 4 in Fig. 25. The numbering of the patients refers to Tables XVI and XVII. If treatment had been continuous for all patients the height increment during the first year varied between 60 and 160 mm with a mean of 105 mm. For the second year the variation was 75—120 mm with a mean of 92 mm. Mean height increment for the following two years was for the two patients studied about 60 mm per year. No side effects to the treatment were found.

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The four patients with doubtful hypopituitarism (Cases 17, 22, 23 and 25, Tables XVII and XXI) did not increase their growth velocity during the treatment. It was continued for at least 6 months since seasonal variations and transient growth accelerations may influence the results if shorter periods are studied.

Antibodies to HGH were not detectable in any patient. Six patients have had treatment for more than 1 1/2 years and two for almost four years.

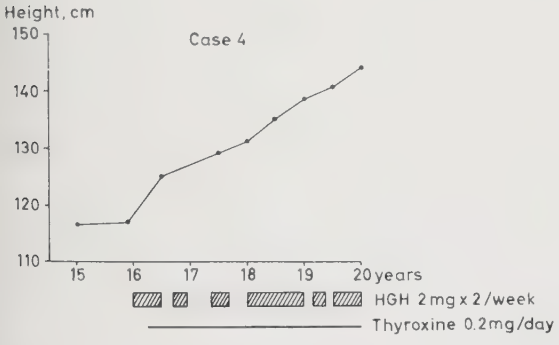

Fig. 25. Growth during four years of treatment with HGH.

Discussion

The treatment with HGH Roos has accelerated the growth in all patients with hypopituitarism. During the first year of treatment the growth velocity was above normal for age except for one case (no. 10) who was of normal height when the treatment began. During prolonged treatment the growth velocity decreased to a level comparable to normal growth. This should not be considered as a failure of treatment since similar growth response is seen after thyroid treatment for hypothyroidism (Talbot and Sobel 1948). The height increment during treatment in this study is of the same order as reported by others (Aarskog 1963, Raben 1964, Seip and Trygstad 1966, Tanner and Whitehouse 1967 and Prader et al. 1967). The dose of HGH used for treatment in the present study is less than the dose used in most other studies but comparable with that in the study of Rosenbloom (1966) where weekly injections of 2.5 to 5 mg were found to be effective in most patients. The immunoreactive activity of HGH Roos is slightly higher when compared to that of other preparations used for treatment and the same may be true of the biological activity as well. The absence of growth improvement in the four children of doubtful pituitary deficiency is in agreement with earlier attempts to treat other types of dwarfism. It is taken as an indication that the reason for stunted growth in these four patients is not pituitary deficiency.

The most encouraging observation in the present study is the absence of antibodies to HGH.

Using HGH Raben (1959), Prader et al. (1967) reported the development of antibodies and the attendant failure to growth in eight of eighteen patients. Tanner and Whitehouse (1967) also found antibodies to HGH Raben in four of nineteen patients. Most patients developed antibodies within a year of treatment, one patient after about 1 1/2 years of treatment. The patient studied by Frasier and Smith (1966) developed antibodies to HGH Li and Papkoff (1956) within nine months of treatment and Parker et al. (1966) reported development of antibodies to HGH Wilhelmi (1961) in one of thirteen children after seven months of treatment. The risk for development of antibodies after the first year of treatment seems to be negligible. In all studies above the patients who developed antibodies became resistant to further treatment with HGH. However, Roth et al. (1964) were able to demonstrate antibodies in three of four patients with long-term treatment with HGH but there was no obvious resistance to HGH therapy. Kaplan et al. (1968) demonstrated HGH antibodies of low binding capacity in one-third of the patients treated for more than six months. The antibodies apparently did not affect the growth rate during treatment. A possible explanation to these differences in the effect of HGH

antibodies could be differences in the antibody titre. A comparison can be made with insulin since most patients develop antibodies to insulin with low binding capacity and without interference of response to treatment.

The absence of antibodies in the present study is in agreement with the results of Seip and Trygstad (1966). They were not able to detect antibodies to HGH Roos in two patients after treatment for $3^1/_2$ years. The presence of antibodies was not tested in the other ten patients treated, but none developed resistance to the HGH treatment.

The reason for the finding that HGH Roos probably does not provoke the development of antibodies is not clear. No available HGH preparation is homogeneous, which has been discussed in Part I for HGH Roos. However, the preparation procedure of Roos et al. is very mild, the pituitaries are initially frozen and then the material is handled only in aqueous solutions at near neutral pH and all steps are performed at 0—4°C. The extraction in glacial acetic acid and the heating to 70°C in the Raben prodedure may partly denature the hormone. Such a conformational change would be a likely explanation to the observed antigenicity.

Summary

Two mg HGH has been given twice a week to nine patients with pituitary dwarfism and to four children in whom pituitary dwarfism was suspected. All nine patients with proved hypopituitarism increased their growth velocity, the mean height increment during the first year of treatment being 10.5 cm. During the following years the growth velocity approached normal growth. None of the patients became resistant to the treatment and regular tests for antibodies were negative. None of four patients with doubtful hypopituitarism responded to the treatment.

TABLE XXIII. *Results of treatment with human growth hormone*

Case	Sex	Age at start of treatment, Years/Months	Growth velocity, mm/year before HGH	Growth rate mm/Months of treatment 1st year	2nd year	3rd year	4th year	Additional therapy	Development of antibodies
2	m	4/11	40	85/12				Thyroxine	ND
3	f	8/2	47	110/12	60/8			Thyroxine	ND
4	m	15/5	18	100/9	80/8	75/12	60/9	Thyroxine	ND
5	m	16/1	11	80/12	60/10			—	ND
6	f	18/1	8	105/12	75/12	40/12	45/12	Thyroxine	ND
7	m	25/2	<5	115/12	60/8			Thyroxine	ND
8	f	8/11	10	90/8				Vasoprestin	ND
9	m	11/0	8	40/3				**	—
10	m	11/3	<5*	30/9	50/8			**	ND
17	m	9/0	40	22/6					ND
22	m	13/7	40	15/6					ND
23	m	13/7	44	27/6					ND
25	m	18/0	22	12/7					ND

ND not detectable.
*Growth during six months between hypophysectomy and start of HGH treatment.
**Operated for craniopharyngioma. Additional theray: Vasopresson, Cortisone and Thyroxine.

CHAPTER XIV

Growth hormone levels in children during long-term treatment with corticosteroids

The administration of corticosteroids has long been known to interfere with normal growth (Blodgett et al. 1956). However, little is known about the mechanism of the growth inhibition. Since the radioimmunoassay for growth hormone made it possible to study the hormone levels in various conditions some studies of the levels during corticosteroid treatment in adults have been published. Hartog et al. (1964 b) studied seven patients with corticosteroid treatment and three patients with Cushing's syndrome. They were not able to show any differences in fasting levels of growth hormone. The increase in growth hormone after insulin-induced hypoglycaemia, however, was diminished for both groups of patients when compared to normal subjects. Frantz and Rabkin (1964) were able to show the same for 11 adult patients treated with long-term steroid therapy in various doses. Furthermore, they demonstrated that the increase in growth hormone levels was less in patients receiving more than 60 mg of Cortisone or equivalent a day. Treatment for two days in a dosage of 300 mg Cortisone a day gave a moderate inhibition in growth hormone response. As for children only a small series of five patients currently under long-term corticosteroid treatment has been published (Root et al.

TABLE XXIV. *Clinical data on 17 children with long-term treatment with corticosteroids*

					Treatment with Cortisone equivalents			
Case	Sex	Age, Years/Months	Delay in bone age, Years/Months	Height increment per centile, previous year	Duration, Years/Months	Total dose, gram	Mean dosage, mg/day previous year	Mean dosage, mg/day previous month
1	f	6/3	—3	<3	3/8	31.3	19.0	19.0
2	m	7/1	—1/3	<3	2/0	12.2	28.1	28.1
3	m	9/0	0	25	5/6	19.3	12.5	—*
4	f	9/6	0	50	4/2	10.8	5.5	5.5
5	m	10/0	—1/6	<3	2/6	31.3	56.5	54.5
6	m	10/6	—0/6	10	7/6	59.2	25.0	23.6
7	f	11/4	0	85	3/6	13.0	—**	—
8	f	11/5	—3/6	<3	8/6	64.7	55.4	35.0
9	f	11/6	—0/6	25	1/0	11.0	30.0	5.5
10	f	12/0	0	8	4/0	28.0	21.0	21.3
11	m	12/0	—2/0	<3	6/0	45.6	30.0	35.9
12	m	12/4	—1	<3	5/1	13.7	30.0	30.0
13	f	13/0	0	10	3/0	18.8	23.1	16.8
14	f	13/1	0	10	1/0	6.9	20.7	13.0
15	m	13/6	0	<3	4/6	18.3	31.0	42.9
16	m	14/1	—1	40	7/5	42.8	12.5	12.5
17	f	15/4	0	50	3/0	21.8	10.0	—***

*Treatment ended 4 months before investigation
**Treatment ended 16 months before investigation
***Treatment ended 6 months before investigation

1967). The diagnosis varied in all five patients and the results are, for several reasons, difficult to evaluate.

Material and methods

Subjects. Seventeen children 6 to 15 years old and with steroid treatment for 1—8½ years were investigated. The reason for steroid treatment was in all patients asthma. All patients were in a good physical condition during the investigation and none had suffered a serious asthma attack during the week immediately preceding the study. The dosage of steroids varied during the last year of treatment but the mean dosage was calculated to allow a comparison with the growth increment during the year. Steroid preparations used were Prednisone, Prednisolone and Triamcinolone but all doses are calculated in Cortisone equivalents. (5 mg Prednisone/Prednisolone equal to 4 mg Triamcinolone equal to 25 mg Cortisone). Data about the patients are summarized in Table XXIV.

Insulin tolerance test. The test was performed as described earlier (page 39) with the single exception that the last blood sample was withdrawn 75 minutes after the insulin injection.

Measurements of growth. Regular control of standing height was performed 3—4 times a year. The height increments are compared with the whole year velocity standards for height of Tanner et al. (1966). Fig. 26 shows the height increments for case 13.

Bone age estimations were performed by the method of Eklöf and Ringertz (1967). The X-rays were all taken by a staff with long experience of the method, all calculations of the bone age were performed by the same radiologist.

Results

The results are summarized in Table XXV and illustrated in Fig. 27 showing a normal response (case 4) and an inhibited response

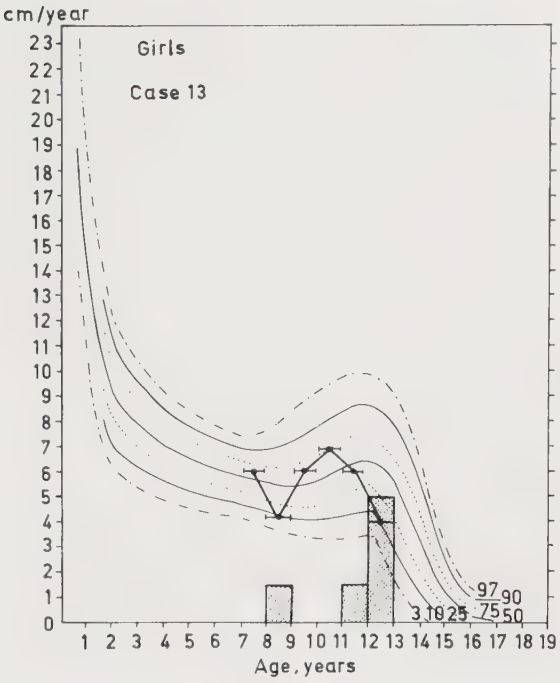

Fig. 26. Growth rate during steroid treatment. Shadowed areas indicate the dosage of Cortisone. A mean dose of 10, 10 and 23 mg Cortisone a day given during the years studied.

(case 11). In all patients the blood glucose dropped to less than 50 per cent of the resting level. Most patients showed minor discomfort during the hypoglycaemia (mild dizziness and sweating) but the test was not interrupted in any patient.

There was no difference in resting levels of growth hormone found between normal children (chapter XI) and children treated with steroids. The increase in growth hormone levels, however, was inhibited by the steroid treatment. There was a statistically significant difference ($p < 0.005$) for the HGH increments of normal children (29.1 ± 11.5 ng/ml) and those treated with corticosteroids (16.6 ± 8.2 ng/ml). There was no difference in time of growth hormone response since most patients showed maximal increment at 45 minutes, some already at 30 minutes and some at 60 minutes after the insulin gift.

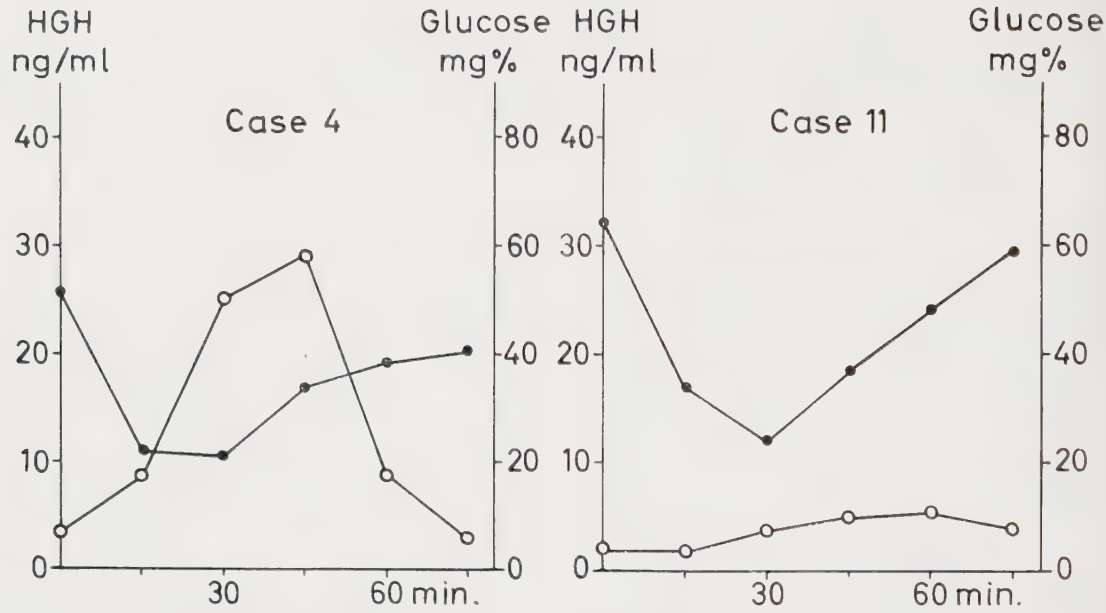

Fig. 27. Blood glucose •—• and HGH o—o response to insulin. Normal response to the left, inhibited response to the right.

A correlation was sought between dosage of corticosteroids the month prior to the investigation and the maximal increment of HGH during insulin load. The result is shown in Fig. 28. The correlation coefficient was —0.66 and the significance for the slope of the regression line to be different from 0 is at $p < 0.005$ level. So there is a significant correlation between dosage of corticosteroids and increment in growth hormone during insulin load.

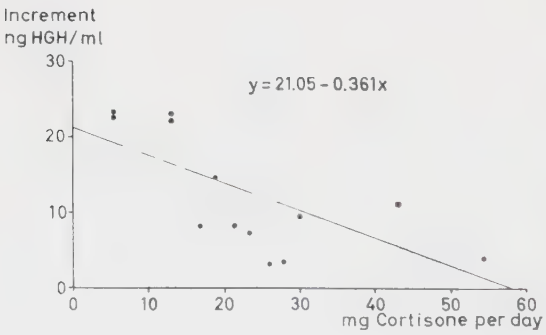

Fig. 28. Correlation between increment of HGH during insulin load and dosage of Cortisone during the month prior to the study. The line indicates the correlation line.

It was possible to find a correlation between height increment percentile and growth hormone increase (Fig 29). The correlation coefficient is 0.52, and the significance for slope of the regression line to be different from 0 is at $p < 0.005$ level. Yet it is very doubtful if this is a true correlation. The height percentile is influenced by the steroid treatment of the previous year while the HGH increment on one occasion never can be representative for the whole year.

Nothing is known about the duration of the inhibition of growth hormone release after the steroid therapy has finished, and how temporal variations in steroid treatment influence the inhibition of growth hormone release. Some data indicate that the inhibition is of rather short duration. If for instance instead of mean Cortisone dose during the last month the corresponding doses for the last three months are studied the correlation coefficient is about —0.32. Furthermore those patients (cases 3 and 17) who recently had finished their treatment showed normal increments. Case 9 having a very high Cortisone dosage (250 mg/day) two months

TABLE XXV. *Growth hormone and blood glucose levels during IV insulin tolerance test in children with long-term treatment with corticosteroids*

Case	Mean Cortisone dosage last month, mg per day	Time in minutes Growth hormone levels in ng/ml Blood glucose levels in mg/100 ml					
		0	15	30	45	60	75
1	19	3.5 50	8.5 35	15 27	18 39	16 50	15 59
2	28	4.5 78	5.5 34	9 31	8 54	5.5 64	2.5 78
3	—*	5 86	4.5 31	9 40	18 49	28 64	10 72
4	5.5	3.5 52	8.5 22	25 21	29 34	9 38	3 41
5	54.5	2 65	2 22	6 19	5 38	6 60	4 76
6	23.6	<1 69	1 34	8 23	8 58	5 73	4 69
7	—**	2 72	8.5 44	50 36	38 58	18 69	5 75
8	35	1 67	1 39	3 20	15 20	13 36	8 55
9	5.5	3.5 80	4.5 37	13 41	26 62	22 78	12 81
10	21.3	4 50	4 29	12 25	9 38	4 59	1 72
11	25.9	2 64	2 34	3 24	5 37	5 48	4 59
12	30	5.5 95	12 60	17 42	15 59	14 86	8 98
13	16.8	6 90	7 36	12 30	14 59	7.5 78	2 84
14	13	11 63	16 30	26 33	34 44	16 73	14 81
15	42.9	5 80	5.5 61	10 38	16 58	14 78	9 80
16	12.5	4 68	3.5 32	19 38	25 51	22 63	18 65
17	—***	7 82	10 47	35 37	33 33	27 72	25 78

*Treatment ended 4 months before investigation
**Treatment ended 16 months before investigation
***Treatment ended 6 months before investigation

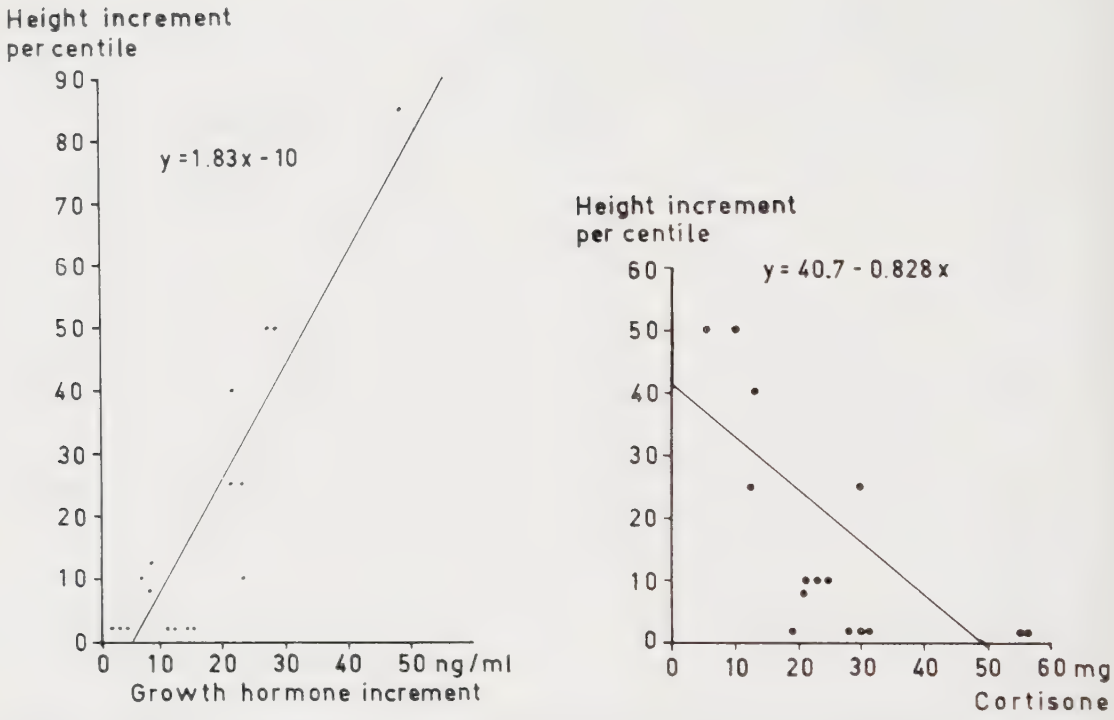

Fig. 29. Correlation between height increment percentile and HGH response to insulin-induced hypoglycaemia to the left, and correlation between height increment percentile and mean Cortisone dose per day during the actual year to the right. The lines indicate correlation lines.

before the investigaion and a moderate dosage at the time of the experiment, shows normal growth hormone increment. It is also noted that growth inhibition is decreased if the corticosteroids are given intermittently.

It would be of interest to try to correlate the mean dosage of Cortisone during the last year with the height increment during that time. This is shown in Fig. 29. The correlation coefficient is —0.68 and the significance for the slope of the regression line to be different from 0 is at $p < 0.005$ level. Furthermore it is noted that those patients who have got a mean Cortisone dosage of more than 20 mg per day during the current year, have a height increment percentile of 10 or less.

The bone age was delayed in 9 of the 17 patients. Roughly all patients with delay in bone age have had a mean Cortisone dose of 7 g or more per year. One patient, however, (case 12) shows a moderate delay in bone age with only 2.7 g Cortisone/year, another patient (case 17) got 7.3 g per year without retardation of the bone age. However, that patient was 15 years old and had finished her steroid treatment six months before the X-ray for the bone age determination.

Discussion

The results of the present study are not in agreement with those found in children by Root et al. (1967), who were not able to show any inhibition in growth hormone release during insulin-induced hypoglycaemia in four of their five Cortisone-treated patients. Results of the method of Root et al. (1967) differ from the results of other methods in the measurement of HGH response to hypoglycaemia in normal children, as well. The reason for this has been discussed earlier (page 40). It is supposed that the

decreased range in normal variation in the technique used by Root et al. will hide the moderate inhibition that would be expected for the comparably low dosage of corticosteroids used in the four patients in which no inhibition could be noted. In accordance with the previous findings of Frantz and Rabkin (1964) and Hartog et al. (1964 b) on adults, the results of the present study show that corticosteroids inhibit the release of growth hormone during insulin-induced hypoglycaemia even in children. The degree of inhibition is correlated to the amount of steroids.

At this time, however, little is known about the mechanism of the inhibition of the pituitary function by the corticosteroids. Vernikos-Danellis (1965) has studied the corticotrophic function in rats and was able to show that Cortisone reduced the corticotrophin-releasing factor (CRF) of the median eminence in the hypothalamus. Furthermore it was shown that a dose of corticosteroids that was sufficient to inhibit stress-induced ACTH-release did not affect the pituitary ACTH content. Whether the suppression is caused by influence on the regulatory system of the hypothalamus or on the synthesis of CRF is unknown.

Corresponding studies in the same animal for GH have been performed by Pecile and Müller (1966) who could show that the content of GH in the anterior pituitary was influenced by corticosteroids, whereas the amount of growth hormone-releasing factor was decreased. Thus even for GH the inhibition seems to be due to some changes in the neurohumoral structures of the hypothalamus. This is in agreement with the findings of Abrams et al. (1964) who demonstrated an impairment in GH response to hypoglycaemia in squirrelmonkeys with hypothalamic lesions and with the results of Roth et al. (1963 a) who, in a patient subjected to pituitary stalk section, found that the usual response to hypoglycaemia was inhibited despite normal resting levels of growth hormone.

In most children with long-term steroid treatment a retardation of the start of puberty is noted. An attractive explanation for this is inhibition in further neurohumoral functions in the hypothalamic region caused by the steroids.

Resting levels of HGH are normal even in steroid-treated children and the inhibition of HGH release during hypoglycaemia, a rather unphysiological state, is not proof that this inhibition is the only or the main reason for the reduced growth in these children. Probably the effect of steroids on the hypothalamic structures is one of several factors that cause the inhibition of growth. One factor could be the katabolic action of cortiocosteroids *per se* resulting in a supply of protein insufficient for adequate growth. The delay in bone age could support this. Of great interest are the results of Friedman and Strang (1966) who found that the growth impairment caused by corticosteroids could be diminished if the patients were treated with a combination of corticosteroids and corticotrophin. Converting the total amount of corticosteroids to corticotrophin did enhance rather than inhibit growth. Since ACTH *per se* does not stimulate HGH secretion, the authors suggest other, presently unknown adrenal steroids released by ACTH, to be growth promoting factors. Several substances as yet not specifically characterised, but with typical steroid color reactions have been found in adrenal venous blood of dogs (Farrel and Lamus 1953). In practice it would look attractive to replace treatment with corticosteroids with ACTH. However, only ACTH of animal origin is avialable and it has to be given in injections. Allergic reactions have been reported but are less common when highly purified ACTH is used. A synthetic α1—24-corticotrophin (Synacten^{R} Ciba) has been introduced and this might eliminate the risk for allergic side-reactions. It would be of interest to study growth hormone increments in children treated with corticotrophin.

Summary

The growth hormone increment induced by hypoglycaemia has been studied in 17 children treated with corticosteroids. No difference in fasting levels was found between normal children and Cortisone-treated children. An inhibition in HGH release due to insulin-induced hypoglycaemia was found as well as a correlation between the inhibition of HGH release and dosage of corticosteroids. Furthermore a correlation between mean dose of corticosteroids per year and height increment was observed. The bone age was delayed in nine of the children all of them having high doses of steroids. The mode of the inhibition to HGH release is discussed. Reduced growth is most likely due as well to factors other than inhibition of HGH release. Despite the disadvantage in method of administration, ACTH might be tried more frequently.

CHAPTER XV

Growth hormone levels during the first week of life in full-term normal infants, infants of diabetic mothers and in premature infants

Several authors have reported high levels of growth hormone during the neonatal period (Cornblath et al. 1965, Laron et al. 1966 and Milner and Wright 1966). However, most of the data are single determinations on different patients during the actual period. Differences have been shown between premature and full-term infants (Cornblath et al. 1965) and between normal infants and infants of diabetic mothers (Laron et al. 1967). The effect on HGH-levels during insulin or glucose load has been reported in isolated cases. Milner and Wright (1966) have reported the growth hormone response to hyperglycaemia induced by exchange transfusion with glucose and disodium citrate used as preservative in twentytwo cases. However, exchange transfusions induce many other metabolic changes in the blood so the results are difficult to evaluate. In this study full-term normal infants and infants of diabetic mothers have been studied with insulin and glucose load during the first week of life. In addition, a small group of premature infants will be presented.

Material and methods

Data about the patients studied are shown in Table XXVI. Prematurity is defined as birth weight below 2,500 g.

All infants were well at time of the study. The reason for the caesarean section in the control infants was threatening obstetrical complications. There was no prolonged labor prior to the operation. The infants of diabetic mothers were routinely delivered by caesarean section.

For the study the umbilical vein was catheterized with a small polyethylene tube (Bardic^{R} infant feeding tube. C. R. Bard, Inc., Murray Hill, U.S.A.). Blood samples were obtained from the tube and placed in glass tubes containing heparin (Vitrum, Stockholm, Sweden). Blood for glucose estimation was withdrawn immediately. The samples were kept at +4°C until centrifuged. The plasma was withdrawn and frozen. To prevent clotting in the polyethylene tube 2 ml of 0.9 % saline with 10 U heparin per ml was injected after the blood sample was drawn. The first 1 ml containing blood, saline and heparin was discarded at each time interval before a blood sample was obtained.

Hypoglycaemia was induced by giving insulin intravenously through the plastic tube in a dose of 4 U/square meter body surface. A blood sample was drawn prior to the insulin load and the insulin was followed by 5 ml of 0.9 % saline. Then the polyethylene tube was replaced by a new one to avoid contamination. Blood samples were drawn every 15 minutes until 105 or 120 minutes after the insulin injection except for some of the prematures where it was impossible to withdraw such large volumes of blood.

Hyperglycaemia was induced by the injection of 0.5 g glucose/kg body weight in 15 % solution. The same precaution of changing the polyethylene tube was taken. Blood samples were collected prior to the glucose injection and at time intervals as above after injection.

The combined insulin-glucose-load was started as an ordinary insulin load, a blood sample was drawn 70 minutes after the insu-

TABLE XXVI. *Clinical data about the patients*

Case number	Sex	Birth-weight, g	Length, cm	Gestation, weeks	Group of patients	Delivery	Investigation performed
1	f	3580	49	40	normal infant	caesarean section	insulin load
2	m	3210	49	39			
3	m	2560	46	38			
4	f	2740	47	39			
5	f	3860	51	40			
6	m	3170	50	40			
7	m	3370	50	38			
8	m	3030	48	38			combined insulin-glucose load
9	m	3910	53	41			
10*	f	3860	51	38			
11	f	2530	47	36	infant of diabetic mother		
12	f	3550	50	37			
13	m	2560	47	36			
14	m	3390	50	36			
15	f	2990	46	37			
16	m	3800	48	36			
17	m	2570	44	36			
18	f	2860	47	36			
19	f	3010	46	36			
20	f	2980	48	38	normal infant	spontaneous	insulin load
21	m	3570	51	40			
22	m	3910	52	40			
23	m	2750	47	38			glucose load
24	f	3100	49	41			
25	f	3880	50	40			
26	f	1570	41	33	premature		insulin load
27	m	1690	43	33			
28	f	2430	46	36			
29	f	1810	44	34			glucose load
30	m	2160	45	35			

*The results obtained show that the patient should be classified as an infant of a mother with gestational diabetes.

lin injection, followed by 0.5 g glucose/kg of body weight within 1 minute. The polyethylene tube was replaced by a new tube and blood samples were collected at 5, 20, and 35 minutes after the glucose gift.

The first investigation was performed between 5—24 hours of age and a second on the same patient at the age of 5—6 days. The age of the infant is the time (hours or days) that has passed since the delivery. The infants were starving before the first investigation and had been fasting about 4 hours prior to the second. To prevent infection the infants got Ampicillin^R in doses of 50 mg/kg and day for 5 days after each umbilical catheterization. Most infants got a small transfusion of blood after the second investigation. No complications to the study have been observed. During the study the temperature of the infant decreased slightly. The decrease, however, did not exceed 1°C.

Blood glucose was estimated by a modification of the glucoseoxidase method using 0.025 M NaOH and 10 % $ZnSO_4$—7 H_2O as protein precipitaing agents. (Hjelm and de Verdier 1963.)

Radioimmunoassay of growth hormone was performed by the method described in Part I with one exception. Since rather high levels of growth hormone were expected the amount of plasma in the incubation mixture was reduced from 50 µl to 25µl. Prior to the

buffer-flow chromatography 25 µl of 7 % bovine serum albumin in 0.9 % saline was added to keep constant the amount of carrier protein in the buffer-flow. For the standard curve 50 µl of a mixture of equal parts heparinized plasma from blood donors and albumin as above was added.

Results

The results are shown in Fig. 30—34 and summarized in Table XXVII. Statistical evaluation of the different HGH release determinations is shown in Tables XXVIII, XXIX and XXXI.

The hypoglycaemia induced by insulin was more pronounced in infants of diabetic mothers. Slightly lower levels were noticed during the first day of life for all groups of patients when compared to the end of the first week. The fall in blood glucose from the basic level was of about the same degree on both occasions studied and in all patients (Fig. 35).

The base levels of HGH are, for all patients studied, higher during the first day of life than at the age of 5—6 days. The base levels of infants of diabetic mothers are markedly lower than those of other groups. The decrease between the first day of life and the age of 5—6 days is less marked in premature infants.

TABLE XXVII. *Serum growth hormone levels in different groups of patients. Mean and range of resting levels and peak levels*

Group of patients	No. of patients	Age	HGH in ng/ml Mean ±S.D. base levels	Range	Mean ±S.D. levels 45 min.	Range
Normal infants caesarean section	9	5—24h	79.2± 8.5	70—90	159.7±23.1	128—90
		5—6 d.	44.0± 8.9	32—60	91.6±10.8	76—110
Infants of diabetic mothers	9	5—24h	47.8± 4.0	38—62	84.1±15.1	65—110
		5—6 d.	28.4± 6.6	22—37	43.6± 6.6	32— 65
Normal infants spontaneous delivery insulin load	3	5—24h	82.3± 9.7	72—90	162.7±20.1	148—178
		5—6 d.	35.7± 7.3	28—43	76.7± 9.2	68— 87
Normal infants spontaneous delivery glucose load	3	5—24h	81.0± 4.3	78—86	177.0±18.2	165—198
		5—6 d.	31.0± 4.0	27—35	76.3±26.8*	62— 84*
Premature infants insulin load	3	5—24h	77.0±14.1	64—92	190.3± 6.2	186—195
		5—6 d.	60.7± 3.4	56—64	198.0±10.1	188—210
Premature infants glucose load	2	5—24h	79.5± 2.5	77—82	176.0±26.8*	157—195*
		5—6 d.	50.5± 3.5	48—53	152.0±15.5*	141—157*

*Indicates results at 60 min., since highest levels were obtained at that time.

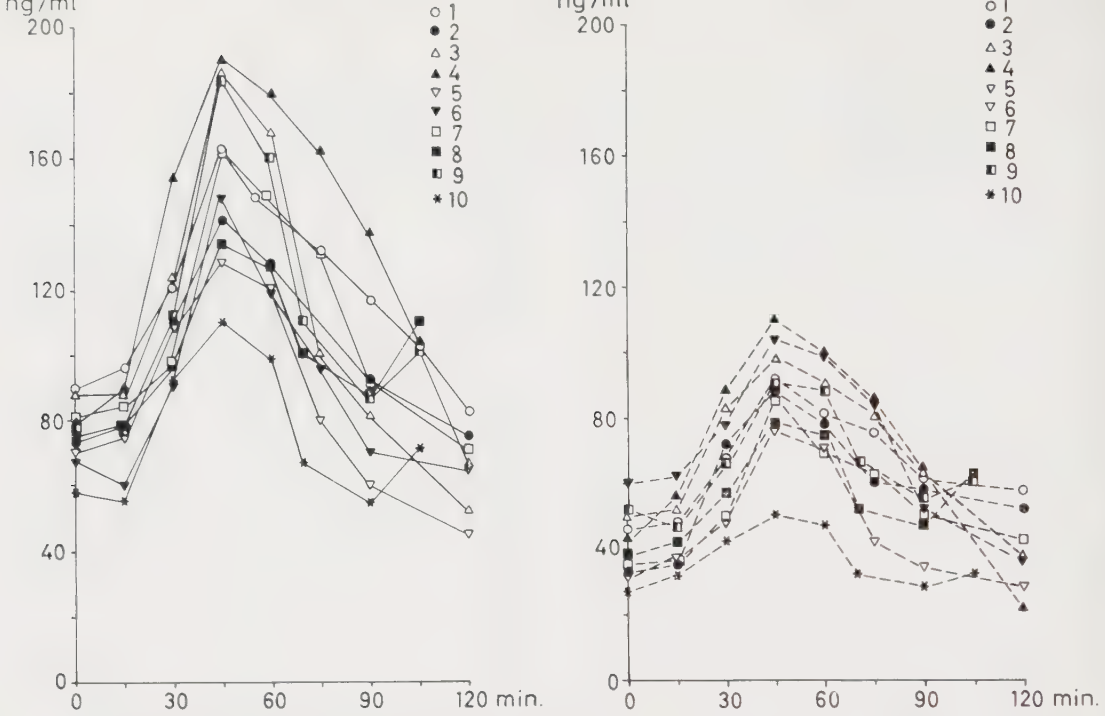

Fig. 30. Growth hormone levels during insulin load on the first day of life —— and at the age of 5—6 days - - - in 10 normal full-term infants delivered by caesarean section. Characters refer to Table XXVI. A combined insulin- glucose load was performed on cases 8, 9 and 10, the glucose being injected immediately after withdrawal of the blood sample at 70 minutes.

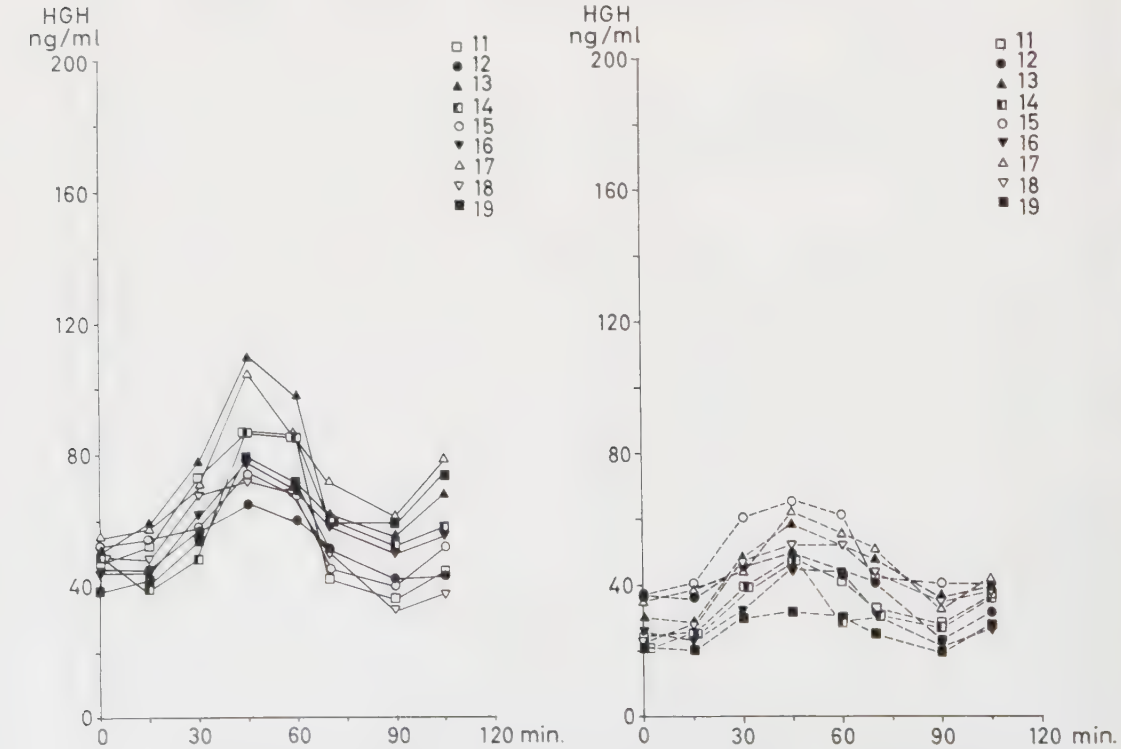

Fig. 31. Growth hormone levels during combined insulin- glucose load on the first day of life —— and at the age of 5—6 days - - - in 9 infants of diabetic mothers. The glucose was injected immediately after withdrawal of the blood sample at 70 minutes. Characters refer to Table XXVI.

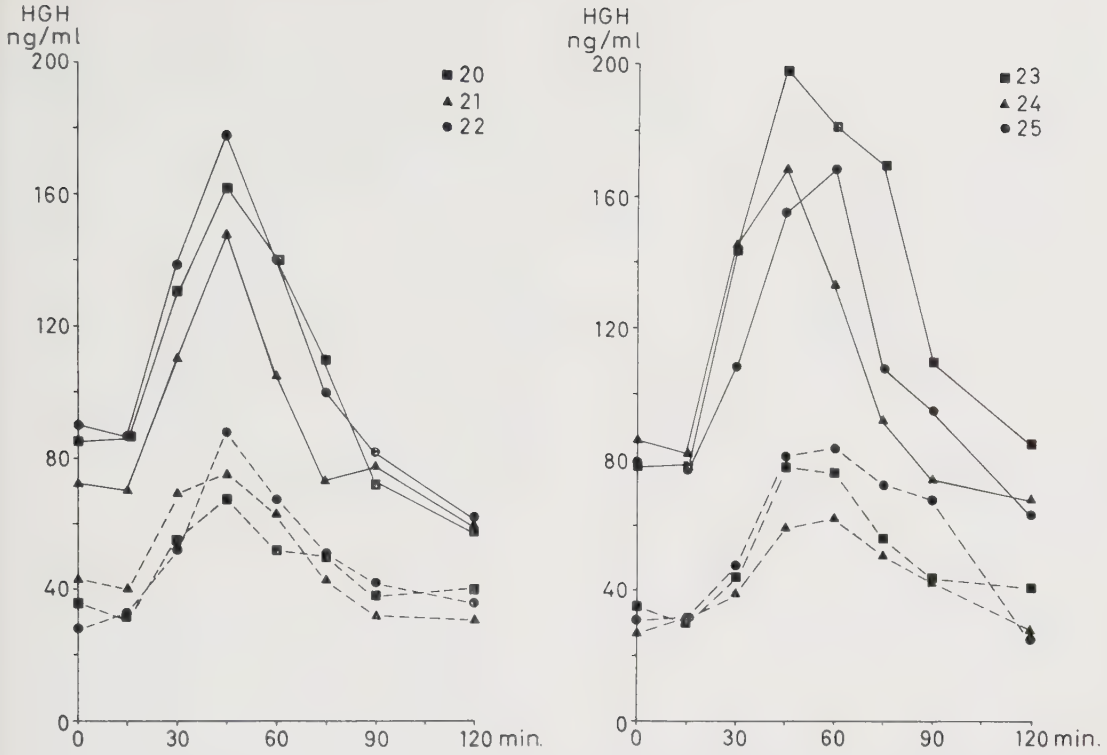

Fig. 32. Growth hormone levels during insulin load (to the left) and glucose load (to the right) on the first day of life —— and at the age of 5—6 days - - - in 6 normal full-term infants delivered spontaneously. Characters refer to table XXVI.

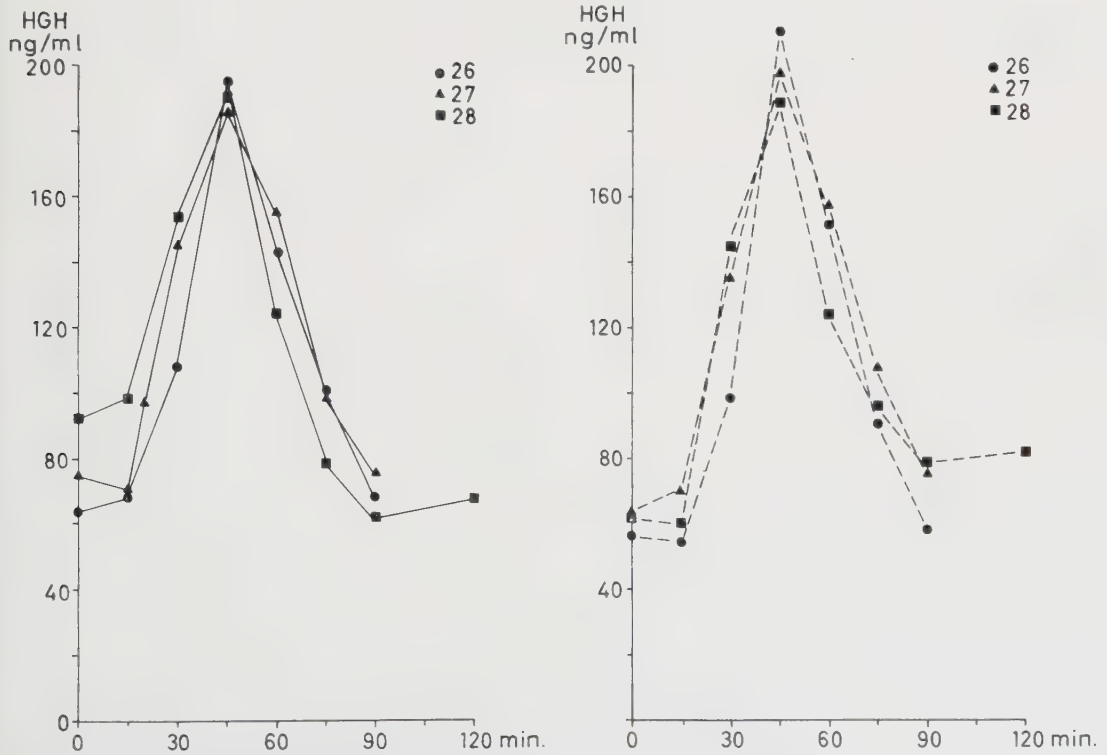

Fig. 33. Growth hormone levels during insulin load in the first day of life —— and at the age of 5—6 day - - - in 3 premature infants. Characters refer to Table XXVI.

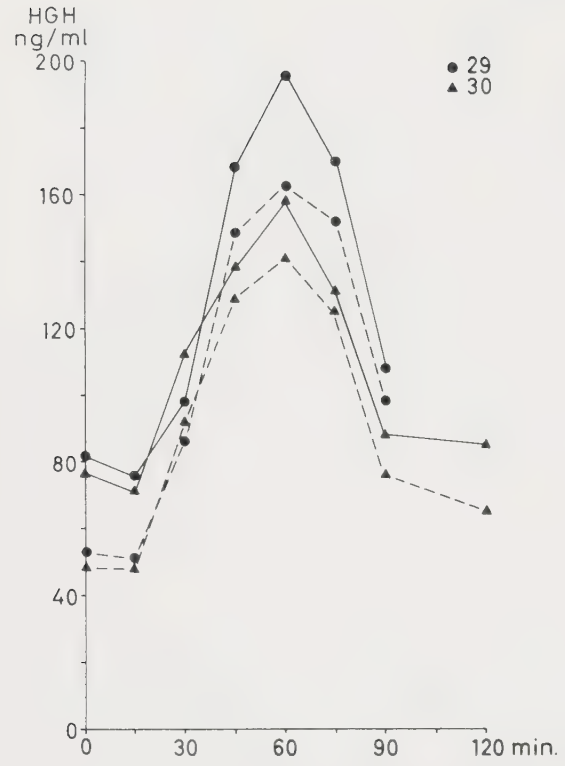

Fig. 34. Growth hormone levels during glucose load in the first day of life —— and at the age of 5—6 days - - - in 2 premature infants. Characters refer to Table XXVI.

The HGH increase due to hypoglycaemia was, when expressed in absolute levels, considerably greater in the first day of life than at the end of the first week for all patients except for the premature infants. The increase was more pronounced in normal and premature infants than in infants of diabetic mothers (Table XXVIII).

If, instead of absolute values of HGH, the per cent maximal increment from the basic levels at the start of the experiment are compared, this increase seems to be of about the same degree for all patients except for the premature infants. These 3 patients increased their increment at the end of the first week when compared to the first day of life. The statistical evaluation is summarized in Table XXIX.

The effect of hyperglycaemia was studied in two different experiments which makes it difficult to compare the patients. The blood glucose levels in the single glucose loads are shown in Fig. 36. The glucose disappearance rate (K_G)* is shown in the figure. It is slightly higher for normal infants than for prematures. In the infants of diabetic mothers the glucose load was added to the insulin load and this was also performed on three normal infants. The rate of glucose disappearance (K_G) for these patients is summarized in Table XXX.

The HGH levels increased during hyperglycaemia in all patients, the increase in absolute levels being more pronounced during the first day of life. The increase was higher for premature infants at the end of the first week than for normal infants. Statistical evaluation is shown in the upper part of Table XXXI. If instead of increase in absolute levels the per cent maximal increment is studied the increase seems to be more pronounced at the end of the first week for prematures while other patients studied do not show any difference. The statistical evalution is shown in the lower part of Table XXXI.

Of special interest is case number 10. Her HGH levels during the whole study are in better agreement with those of infants of diabetic mothers than with those of normal infants. She is the second child of the mother, the first child having a birth weight of 4,680 g. A glucose tolerance test on the mother one month after delivery of the first child showed a glucose disappearance rate (K_G) of 1.01.

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* K_G according to the method of Hamilton and Stein (1942) as modified by Ikkos and Luft (1957).

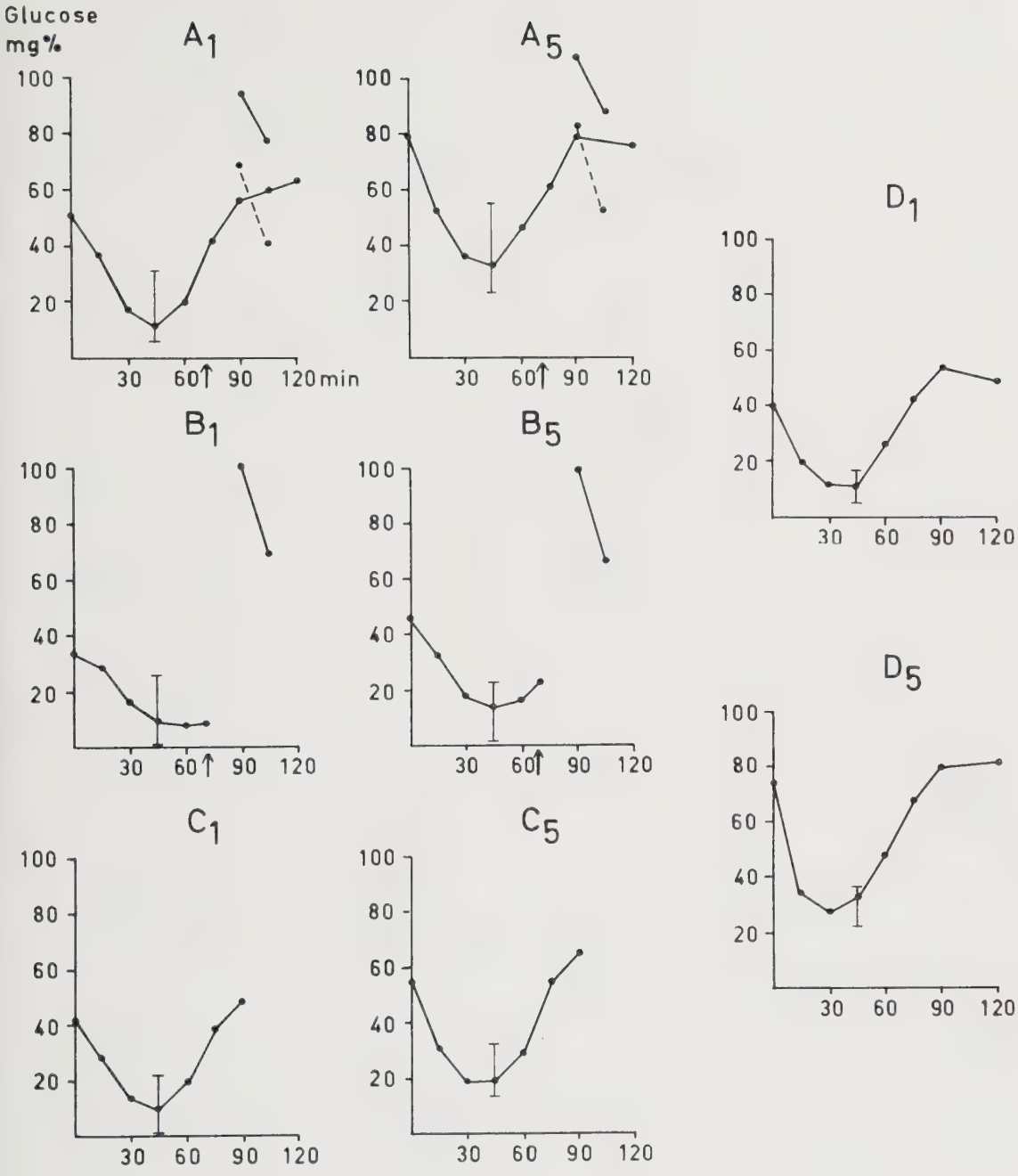

Fig. 35. Mean of blood glucose during insulin loads. Bars indicate range of lowest levels. The characters 1 and 5 refer to the ages 1 and 5—6 days respectively. The letters A—D refer to the following groups: A: Normal infants, insulin load except for 3 patients with combined insulin-glucose load. - - - indicates case 10. B: Infants of diabetic mothers. C: Normal infants delivered spontaneously. D: Premature infants.

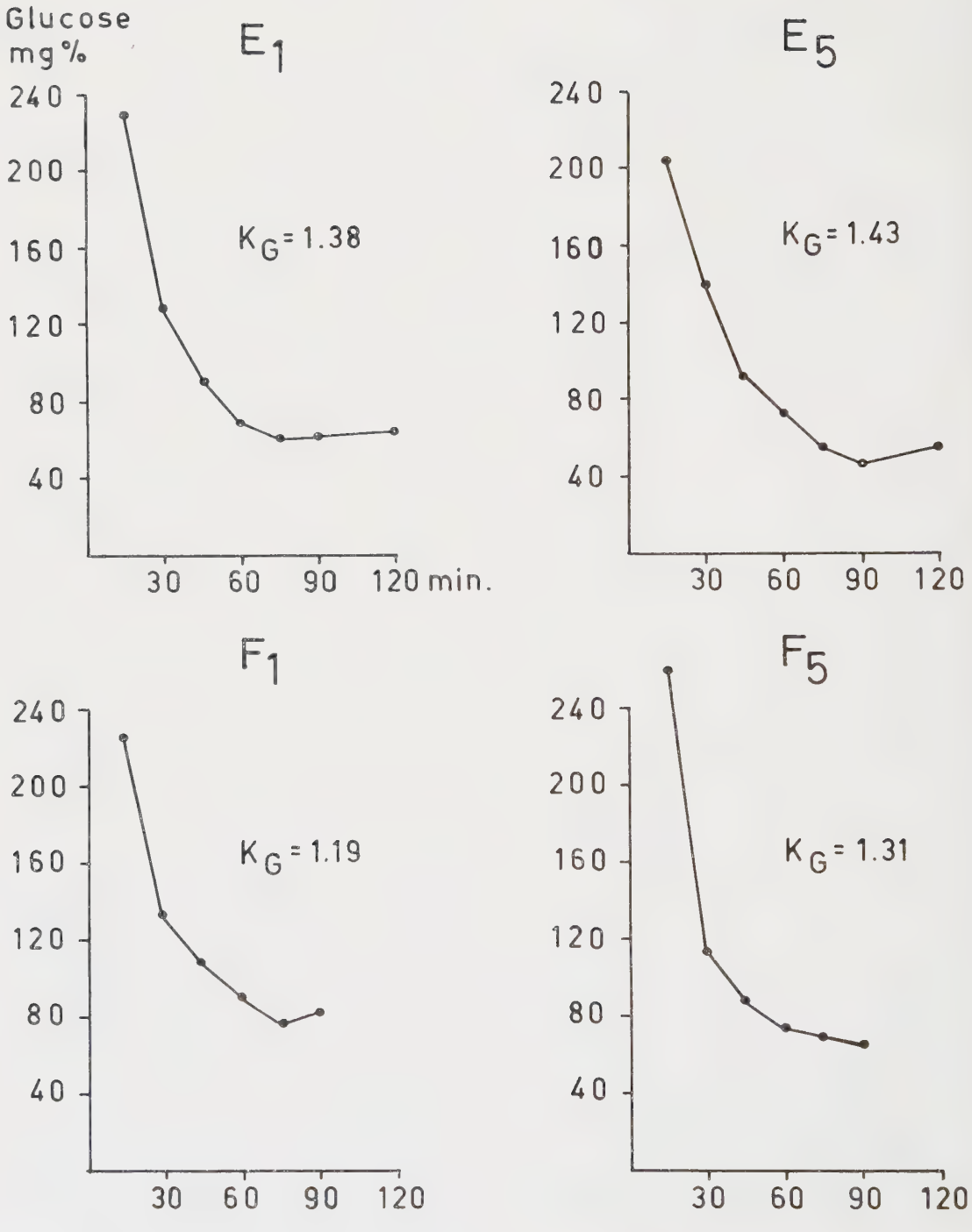

Fig. 36. Mean of blood glucose during glucose load in the first day (1) and at the age of 5—6 days (5). E: Normal infants. F: Premature infants.

The K_G's are calculated as stated on page 68.

The glucose disappearance rate (K_G) for case 10 is shown in Table XXX. The high K_G value for patient 10 and for infants of diabetic mothers is in agreement with the results published by Gentz et al. (1967) and is a further support for the suspicion that case 10 should be classified as child of mother with gestational diabetes. This patient has therefore been excluded from the normal infants in the statistical evaluations.

TABLE XXVIII. *Significance tests for HGH response to insulin-induced hypoglycaemia. Absolute levels at all time-intervals studied. IDM stands for infants of diabetic mothers*

Groups of infants compared			p-value
Normal 1:st day	>	Normal 5—6 days	<0.001*
IDM 1:st day	>	IDM 5—6 days	<0.001*
Normal 1:st day	>	IDM 1:st day	<0.001**
Normal 5—6 days	>	IDM 5—6 days	<0.001, except 70 min. p <0.01**
Premat. 5—6 days	>	Normal 5—6 days	<0.005, except 15 min. p <0.03**

*Differences for each patient as compared by Student's t-test for paired samples at all time intervals studied.
**Mean level of HGH for each time interval studied in one group as compared by a Student's t-test for non-paired samples with corresponding levels of the other group.

TABLE XXIX. *Significance tests for HGH response to insulin-induced hypoglycaemia. Per cent increment of base level is compared. In the upper part of the table no statistically significant differences are present in contrast to the lower part. IDM stands for infants of diabetic mothers*

Groups of infants compared			p-value
Normal 1:st day	>	Normal 5—6 days	>0.2*
IDM 1:st day	>	IDM 5—6 days	>0.2*
Normal 1:st day	>	IDM 1:st day	>0.1**
Normal 5—6 days	>	IDM 5—6 days	>0.08**
Normal 1:st day	>	Premat. 1:st day	>0.1**
Premat. 5—6 days	>	Premat. 1:st day	<0.025*
Premat. 5—6 days	>	Normal 5—6 days	<0.001**

* and ** see Table XXVIII

TABLE XXX. *Glucose disappearance in combined insulin-glucose load. The values are calculated from the glucose levels 20 and 35 minutes after the glucose gift. IDM stands for infants of diabetic mothers*

Group of infants	No. of patients	Age	K_G
Normal infants	2	1:st day	1.32
Normal infants	2	5—6 days	1.46
IDM	9	1:st day	2.51
IDM	9	5—6 days	2.75
Patient no 10		1:st day	3.51
Patient no 10		5—6 days	3.29

TABLE XXXI. *Significance tests for HGH response to hyperglycaemia. Absolute levels at all time-intervals studied are presented in the upper part. Per cent increment of base level is compared in the lower part. IDM stands for infants of diabetic mothers*

Groups of infants compared			p-value
Normal 1:st day	>	Normal 5—6 days	<0.001*
IDM 90 min. 1:st day	<	IDM 105 min. 1:st day	<0.001*
IDM 90 min. 5—6 days	<	IDM 105 min. 5—6 days	<0.001*
Premat. 5—6 days	>	Normal 5—6 days	<0.025 except for 90 min $p<0.1$**
Normal 1:st day	>	Normal 5—6 days	>0.2*
IDM 1:st day	>	IDM 5—6 days	>0.2*
Normal 1:st day	>	Premat. 1:st day	>0.1**
Premat. 5—6 days	>	Premat. 1:st day	<0.05*
Premat. 5—6 days	>	Normal 5—6 days	<0.025**

* and ** se Table XXVIII

Discussion

The paucity of data on HGH levels in the neonatal period and the wide variations in individual HGH response make the evaluation of the results difficult.

The high base levels during the first day of life were initially believed to depend on placental transfer of human placental lactogen (HPL), a substance with hormonal activity present in maternal serum in amounts that at term are about 1,000 times those of HGH (Kaplan and Grumbach 1963). HPL crossreacts to some degree with HGH antiserum and would in part be measured in the immunoassay as HGH. However, Beck et al. (1965) have shown with a specific immunoassay for HPL that cord blood contains hardly measurable amounts of HPL.

Studies of HGH response to hypoglycaemia have been performed earlier in only four normal infants and two premature infants during the first day of life (Cornblath et al. 1965).

The results are in agreement with those found in the present study. No infants of diabetic mothers have been studied and it is interesting that gestational diabetes seems to provoke changes in HGH response similar to those found in infants of diabetic mothers.

Hyperglycaemia has been studied in eight infants with a continuous glucose infusion (Cornblath et al. 1965) and during exchange transfusion by Milner and Wright (1966). Thus the experiments are not quite comparable. However, all studies have shown an increase in HGH levels to hyperglycaemia during the neonatal period contrary to the findings at the age of 15 days or more (Cornblath et al. 1965).

The role of growth hormone during the neonatal period is not clearly understood. It does not seem to be indispensable as a growth promoting factor during the neonatal period since anencephalic human infants, even when born without a pituitary gland, have large, well-proportioned bodies (Seckel 1960). Furthermore the administration of growth hormone in premature infants does not accelerate the bone growth (Chinmello et al. 1965).

The finding that HGH levels in newborn infants are higher than those of the mother (Laron et al. 1966) indicate an active secretion of hormone by the foetus. Kaplan and Gumbach (1962) have demonstrated growth hormone in the pituitary gland of a 15 week foetus. The disappearance of growth hormone was studied (Cornblath et al. 1965) by intravenous administration of the hormone to newborn infants. The average $T^1/_2$ was $12^1/_2$ minutes compared with about 25—30 minutes for adults (page 29). So the high values obtained during the neonatal period are not due to slower plasma clearance.

For adults the main function of growth hormone seems to be homeostasis of energy-providing substrates by its lipolytic action. If the energy requirements can be met by carbohydrates, secretion of growth hormone is not demanded. Glick et al. (1965) demonstrated that the normal prompt rise in HGH to insulin-induced hypoglycaemia was prevented by a simultaneous administration of glucose. The subsequent fall in blood glucose after glucose administration led to a rise in HGH which was correlated in time with the secondary rise in plasma free fatty acids (FFA) after an oral glucose load. (Bolinger et al. 1962). There is no correlation between the absolute levels of blood glucose and growth hormone secretion since even very small changes in blood glucose induced by infusion of small amounts of insulin over an hour causes a marked rise in plasma HGH (Luft et al. 1966.). Prolonged fasting, particularly in conjunction with exercise, increases the growth hormone levels (Hunter and Rigal 1966). Even here the effect of growth hormone will be energy homeostasis by lipolysis.

The intravenous administration of large amounts of some amino-acids, particularly arginine, is another stimulus of growth hormone secretion (Knopf et al. 1965). Arginine infusion induces an increase in plasma insulin but the blood glucose levels are not influenced. This indicates a collaboration of insulin and growth hormone in the incorporation of amino-acids into protein, a further physiological activity of growth hormone. The first evidence for the effect of growth hormone on protein synthesis in man were shown by Bech et al. (1957). Growth hormone seems to stimulate the transfer of amino-acids from the extracellular to the intracellular compartments (Knobil and Hotschkiss, 1964).

Whether these two actions of growth hormone, the lipolytic and the protein synthesizing, are due to action of the same molecule, the one-factor hypothesis (Raben 1965), or due to the two-factor hypothesis of Levine and Luft (1964) which suggest that the so called growth hormone of the anterior pituitary is composed of two components, one somatotrophic with the protein synthesizing effect and one adipokinetic with the lipolytic effect, is yet to be proved. According to the hypothesis of Levine and Luft the immunoassay of HGH will measure both effects.

Supporting the two-factor hypothesis are the results of Trygstad (1967) which have been discussed in Part I, page 32.

These fundamental functions of growth hormone can explain most of the results of the insulin loads in this study. The new environment is energy-consuming and blood sugar decreases during the first 24 hours (Cornblath et al. 1961). Person and Gentz (1966) have shown that there is an increase in FFA from rather low levels in cord blood to very high levels within the first 48 hours of life. The high levels remain during the neonatal period. When blood glucose is depressed by insulin, growth hormone, by its lipolytic action, might be the main regulator to energy homeostasis and consequently increases dramatically. When feeding has started the demand for energy from FFA is less important, which would explain the more moderate HGH levels at the age of 5—6 days.

However, there is no difference in per cent maximal increment between the first and the second period investigated, so the lower levels as well could be the result of a successive adaptation to a lower level of growth hormone with increasing maturity of the infant. This also could be an explanation for the results obtained in premature infants, their higher degree of immaturity resulting in a slower adaption. However, for premature infants another factor might be of importance. Blood glucose levels during the neonatal period are lower for prematures compared to full-term infants (von Euler et al. 1964). The main reason for this would be the markedly lower stores of glycogen until the 37th week of gestation (Shelley and Neligan. 1966). The energy demand has to be supplied from other sources, the elevated growth hormone levels raise the FFA levels. For the present weight group Melichar et al. (1964) have shown moderately elevated levels of FFA.

As for infants of diabetic mothers: they have decreased blood glucose levels during the neonatal period (Pedersen et al. 1954), the reason for this being a rapid disposal of glucose from the blood due to hyperinsulinism (Baird and Farquhar, 1962). The FFA in infants of diabetic mothers are lower when compared to those in normal infants (Chen et al. 1965 and Laron et al. 1967), which might be the effect of the functional hyperinsulinism and/or the low HGH values. The most likely explanation for most of the metabolic abnormalities in the infant of diabetic mothers is the maternal hyperglycaemia (Pedersen et al. 1954). The better the control of the diabetes of the mother, the lower the body weight and fewer symtoms of altered metabolism in the child (Pedersen 1967). A possible hypothesis is that the variations in blood glucose during gestation will increase the demands on the regulatory system. The latter might improve and be more mature. Therefore the resting levels would be closer to those found in older infants. Exhaustion of the pituitary or regulary system because of large demands during foetal life would give, beside a low basic level, a depressed response to hypoglycaemia, which has not been shown.

More difficult to explain is the paradoxical increase in growth hormone after induced hyperglycaemia. Cornblath et al. (1965) have shown that this effect remains about 14 days in full-term infants and they have suggested different manners for hypo- and hyperglycaemia to trigger the secretion of growth hormone, and that the pathway for suppression requires time after birth to mature. In all groups of patients in this study (full-term infants, infants of diabetic mothers and prematures) an increase in growth hormone is induced by hyperglycaemia, most pronounced in prematures. This could fit the hypothesis of Cornblath et al. (1965). Another but more venturous possibility is that in newborn infants even a transient hyperglycaemia is a signal for protein synthesis. Growth hormone is necessary for that and protein synthesis and growth are never as intense as in the neonatal period. A support for this hypothesis would be the significantly greater increase in HGH levels induced by glucose load for prematures

at the age of 5—6 days when compared to the first day of life.

If one accepts the two-factor hypothesis of Levine and Luft (1964) there is another possibility. During insulin load the adipokinetic component is produced in high levels to provoke lipolysis. When glucose increases during glucose load the protein synthesizing somatotrophic component increases. It would be of great interest to repeat the estimations with an immunoassay-system for the two components described by Trygstad (1967).

Stress is another factor proved to increase growth hormone levels (Greenwood and Landon 1966). The stress of labor resulting in elevated levels of 17-OH corticosteroids (Migeon et al. 1956) affects neither the basic levels of growth hormone nor the release of growth hormone by insulin load since there are no differences between the infants delivered by caesarean section and those delivered spontaneously. However, the experiment *per se* with the catheterization of the umbilical vein, the moderate lowering of the body temperature and the infusion within 1 minute of a volume which for the glucose load constitutes about 10 % of the blood volume, are stressing factors that might stimulate HGH secretion.

At this time too little is known about factors concerning the neonatal metabolism to give an entire explanation about the fundamental role of growth hormone during this period.

Summary

Growth hormone levels during insulin- and glucose load have been studied in normal infants, infants of diabetic mothers and in premature infants during the first week of life.

Base levels of HGH were elevated in all patients when compared to older infants. The highest levels were present on the first day of life. Lower levels were found in infants of diabetic mothers than in the other patients studied.

Insulin-induced hypoglycaemia increased HGH levels in all patients. The increase was more pronounced at the age of one day as compared to 5—6 days if the absolute levels were compared. The increase was more moderate in infants of diabetic mothers. If the response to hypoglycaemia is expressed as the per cent maximal increment from basic levels no difference in response can be shown between the first day of life and the age of 5—6 days or between normal infants and infants of diabetic mothers. For premature infants the response in per cent increment was more pronounced at the end of the first week and of much higher degree than for other infants studied.

Hyperglycaemia induced an increase in HGH levels comparable to that found during hypoglycaemia. The differences between the groups studied are the same as for hypoglycaemia.

General Summary

PART I

Methodological studies

A radioimmuno-chromatographic assay for human growth hormone (HGH) is described. The method has been carefully tested with special reference to the influence of degradation damage of HGH and to the method's applicability to neonatal plasma. Recovery experiments have been performed and the precision and sensitivity of the method is comparable with other radioimmunoassays for HGH. The method has been tested clinically and the results are comparable with those obtained by similar methods.

The radioimmuno-chromatorographic technique can be used for the detection of antibodies to HGH.

PART II

Clinical studies

1. The HGH release to insulin-induced hypoglycaemia has been studied in 16 normal children and the normal response has been defined as an increase in HGH levels of 10 ng/ml or more.

2. The HGH release in children of short stature has been studied in order to diagnose hypopituitary dwarfism. The HGH release in ten children with proved hypopituitarism was subnormal as defined above. Two of fifteen patients without pituitary deficiency showed subnormal response. Five children with Turner's syndrome and two with chondrodystrophic nanism showed a normal HGH response. The HGH response to the insulin tolerance test has been compared with other available tests for the evaluation of pituitary function.

3. Nine hypopituitary dwarfs have been treated with human growth hormone. The height increment during the first year of treatment varied between 6 and 16 cm with a mean of 10.5 cm. During the second year of treatment the mean was 9.2 cm. No patient became resistant to the treatment and regular tests for the presence of antibodies to HGH were not able to reveal any.

4. Seventeen children with long-term treatment with corticosteroids for bronchial asthma were investigated for HGH release to insulin-induced hypoglycaemia. The response was found to be decreased when compared to normal children and a correlation coefficient of —0.66 was found between dose of corticosteroids and HGH increment.

5. Thirty newborn infants were investigated during the first day of life and at the age of 5—6 days. Normal infants, infants of diabetic mothers and premature infants showed elevated fasting levels and a vigorous increase in HGH levels during hypoglycaemia as well as during hyperglycaemia. The increase was more pronounced during the first day of life, and more moderate in infants of diabetic mothers. There was no difference in per cent increment from base levels between normal infants and infants of diabetic mothers. For premature infants the per cent increment was greater compared to the other two groups and was most pronounced at the end of the first week of life. The results are discussed with references to fat and carbohydrate metabolism during the first week of life.

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